

UNIVERSIDADE DE LISBOA  
FACULDADE DE CIÊNCIAS  
DEPARTAMENTO DE BIOLOGIA VEGETAL



**GENOTYPIC ANALYSIS OF THE *LEISHMANIA*  
*INFANTUM* RESISTANCE TO CONVENTIONAL DRUGS  
AND NEW CHEMICALLY SYNTHESIZED COMPOUNDS**

**David Jorge Santos Mateus**

**DISSERTAÇÃO**

**MESTRADO EM MICROBIOLOGIA APLICADA**

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Dissertação orientada por Prof. Dr. Gabriela Santos-Gomes (IHMT-UNL)  
e Prof. Dr. Francisco Dionísio (FCUL)

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**2014**

This thesis was fully performed at Center for Malaria and Other Tropical Diseases of Institute of Hygiene and Tropical Medicine, New University of Lisbon under the direct supervision of Prof. Dr. Gabriela Santos-Gomes.

Prof. Dr. Francisco Dionísio was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.



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## ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my supervisor Prof. Dr. Gabriela Santos-Gomes. Without her knowledge, support, patience and guidance this dissertation would not have been possible. I am eternally grateful for her willingness to take me into her research team, for the support on the good and bad moments and for being the best supervisor imaginable. She was not only a mentor but also became a good friend.

I would like to thank Prof. Dr. Francisco Dionísio for accepting to be my internal supervisor in this project, for his insightful comments and advices. My thanks to Prof. Dr. Eugénia Cruz and Dr. Manuela Carvalheiro for giving us the new antileishmanial formulations necessary for this research project.

My sincere appreciation to Armanda Rodrigues for the practical orientation given when I first arrived in the laboratory and for lending me “half a brain” once in a while from then on. My thanks also goes for my fellow colleagues Ana Bolas, Cristina Branco, Mafalda Claro, Maria Pereira, Mariana Fernandes and Joana Cavaco Silva. Without all of you and your friendship our laboratory is not the same. I am glad you are always there to cheer up my days.

A special thanks to all my dearest friends, especially to André Soares, Claudio Monterosso, Diogo Pereira, Filipa Marta, Inês Gato, João Robalo, Joana Vale and Miriam Silva. Thank you for being always there for me, for giving me motivation and, most of all, for making my world a better place.

At last, but by no means the least, I would like to give my most heartfelt thanks and appreciation to my mother. Thank you for creating me and for never stop supporting me throughout my life.

This work was funded by the Portuguese Foundation for Science and Technology (FCT) and the European Union (FEDER) through the projects PTDC/CVT/098290/2008 and PTDC/CVT/113121/2010.



## LIST OF ABBREVIATIONS

ACR2 – arsenate reductase 2  
AmB – amphotericin B  
AQP1 – aquaglyceroporin  
bp – base pairs  
CanL – canine leishmaniasis  
CL – cutaneous leishmaniasis  
CoA – coenzyme A  
DMSO – dimethyl sulfoxide  
DNA – deoxyribonucleic acid  
dNTP – deoxynucleotide  
DOTS – directly observed treatment, short-course  
EC<sub>100</sub> – effective concentration to kill 100% of parasites  
EDTA – ethylenediaminetetraacetic acid  
EU – European Union  
FBS – fetal bovine serum  
FCUL – Faculty of Sciences of the University of Lisbon  
FW – forward  
GSH – glutathione  
GSH1 – gamma-glutamylcysteine synthase  
*GSH1* – gamma-glutamylcysteine synthase gene  
HIV – human immunodeficiency virus  
IHMT – Institute of Hygiene and Tropical Medicine  
IP – intraperitoneally  
IPTG – isopropyl  $\beta$ -D-1-thiogalactopyranoside  
LB – lysogeny broth  
LDA – limiting dilution assay  
m/v – mass/volume concentration  
MDR1 – P-glycoprotein MDR1  
*MDR1* – P-glycoprotein MDR1 gene  
MGA – meglumine antimoniate  
MILT – miltefosine  
ML – mucocutaneous leishmaniasis  
MRPA – ABC-thiol transporter MRPA  
*MRPA* – ABC-thiol transporter MRPA gene  
MT – putative miltefosine transporter protein

ORZ – oryzalin  
 PBS – phosphate buffered saline  
 PCR – polymerase chain reaction  
 PEG – polyethylene glycol  
*POL1* – DNA polymerase alpha catalytic subunit gene  
 ppg – per gram of tissue  
 PTR1 – pteridine reductase  
*PTR1* – pteridine reductase gene  
 RNA – ribonucleic acid  
 ROS – reactive oxygen species  
 rpm – rotations per minute  
 RV – reverse  
 Sb<sup>III</sup> – trivalent antimonial  
 Sb<sup>V</sup> – pentavalent antimonial  
 SCHN – Schneider medium  
 SOC – super optimal broth with catabolite repression  
 STB – sodium stibogluconate  
 T(SH)<sub>2</sub> – trypanothione  
 TAE – tris-acetate-EDTA  
 T<sub>AN</sub> – annealing temperature  
 TDR1 – thiol-dependent reductase 1  
 TFL – trifluralin  
 TFL-A – trifluralin analogue  
 TFL-A3 – trifluralin analogue 3  
 TFL-A6 – trifluralin analogue 6  
 TR – trypanothione reductase  
 TS<sub>2</sub> – trypanothione disulfide  
 TSS – transformation and storage solution  
 U – units  
 UK – United Kingdom  
 UNL – New University of Lisbon  
 USA – United States of America  
 v/v – volume/volume concentration  
 VL – visceral leishmaniasis  
 WHO – World Health Organization  
 WT – wild-type  
 X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

## RESUMO

A leishmaniose, doença parasitária com manifestações clínicas diversas, é considerada uma das mais importantes patologias parasitárias. É causada por cerca de 20 espécies de protozoários intracelulares do género *Leishmania* sendo, na maioria dos casos, uma zoonose de mamíferos selvagens ou domésticos que atinge acidentalmente o Homem. Este parasita é transmitido aos hospedeiros vertebrados pela picada de flebotómídeos do género *Phlebotomus* no Velho Mundo e *Lutzomyia* no Novo Mundo. O ciclo de vida deste inclui dois estádios com duas formas morfológicas distintas: forma amastigota no interior dos macrófagos do hospedeiro vertebrado e forma promastigota no intestino do insecto vector. Com base nas suas manifestações clínicas a leishmaniose pode ser classificada em três tipos principais: leishmaniose cutânea, leishmaniose mucocutânea e leishmaniose visceral. *Leishmania infantum* é o agente responsável pela leishmaniose visceral zoonótica, manifestação severa e mortal da doença, que se distribui pela região da bacia do Mediterrâneo incluindo Portugal.

Há mais de 70 anos que os antimoniais pentavalentes ( $Sb^V$ ), como estibogluconato de sódio (STB) e antimoniato de meglumina (MGA), têm sido usados como tratamento de primeira linha em todas as manifestações clínicas da doença. Estes fármacos, apesar de serem bastante eficazes no combate à leishmaniose, apresentam toxicidade elevada, sendo responsáveis por efeitos secundários graves. Nos últimos anos, a sensibilidade do parasita a estes fármacos tem sofrido alterações problemáticas em algumas regiões endémicas para *Leishmania*. Novos fármacos mais eficazes e/ou menos tóxicos têm vindo a ser usados em resposta à diferenciação de estirpes do parasita com menor sensibilidade aos antimoniais ou em situações clínicas de maior gravidade. Porém, o elevado custo torna impossível a utilização regular destes fármacos nos países mais afectados, uma vez que esta doença está frequentemente associada a países de baixa renda. Apesar da elevada quantidade de fármacos anti-*Leishmania* actualmente em uso, nenhum deles conjuga elevada eficiência, baixa toxicidade e custo acessível às populações afectadas. No seu conjunto, estes factores fazem da investigação de novos fármacos anti-*Leishmania* uma prioridade. Actualmente a investigação de novos fármacos está muito focada em compostos existentes com actividade terapêutica dirigida a outras doenças, compostos de origem natural ou formulações de herbicidas. Entre estes últimos compostos encontram-se os derivados de dinitroanilinas, nomeadamente a orizalina (ORZ) e compostos análogos da trifluralina (TFL), tendo o seu potencial no tratamento de leishmaniose sido demonstrado.

A diminuição da sensibilidade do parasita aos fármacos em uso foi relacionada com a habilidade de amplificar selectivamente o número de cópias de alguns genes em

resposta ao contacto com as formulações. A amplificação génica é considerada o principal mecanismo de resistência a fármacos dentro do género *Leishmania*. O pequeno genoma e a reduzida quantidade de genes fazem deste parasita um alvo ideal para o estudo deste fenómeno. A ausência de controlo da transcrição é também um dos factores de sucesso da amplificação génica como mecanismo adaptativo, tendo sido demonstrado que o DNA amplificado pode atingir 10% do DNA total do parasita. Alterações na permeabilidade da membrana, permitindo uma inferior acumulação do fármaco dentro da célula, redução na importação do fármaco, inactivação do fármaco ou sequestro em compartimentos intracelulares são os mecanismos usados por *Leishmania* para sobreviver ao contacto com fármacos anti-*Leishmania*. O aumento do número de cópias dos genes das proteínas responsáveis por estes mecanismos está directamente relacionado com o aumento do respectivo nível de expressão mas também com o aumento da probabilidade de ocorrência de mutações pontuais em algumas destas cópias que poderão vir a ser vantajosas para a sobrevivência do parasita quando em contacto com fármacos.

Neste estudo foi avaliado o potencial anti-*Leishmania* em modelo animal de um fármaco anti-*Leishmania* clássico, o estibogluconato de sódio, da dinitoanilina orizalina e de novos compostos sintéticos análogos da trifluralina, TFL-A3 e TFL-A6, que demonstraram elevada actividade anti-*Leishmania* e baixa toxicidade *in vitro*. Em modelo animal o tratamento não conduz à eliminação completa dos parasitas, tendo-se colocado em hipótese que os parasitas sobreviventes serão menos sensíveis ou resistentes aos fármacos usados. Neste estudo os parasitas que sobreviveram ao tratamento foram quantificados e recolhidos para extracção de DNA e o número de cópias de genes seleccionados foi quantificado por PCR em tempo real. Estudos anteriores efectuados em amostras recolhidas de pacientes de áreas endémicas de leishmaniose demonstraram que a menor susceptibilidade do parasita aos fármacos está relacionada com a amplificação dos seguintes genes: os genes para os transportadores de membrana MRPA (*MRPA*) e MDR1 (*MDR1*), o gene para a enzima gama-glutamyl-cisteína sintetase (*GSH1*), envolvida na síntese de glutathione e tripanotona, e o gene para a enzima pteridina redutase (*PTR1*), que intervém na cascata metabólica do folato. A quantificação absoluta do número de cópias de cada gene por parasita foi possível recorrendo a um gene de cópia única no genoma de *Leishmania*, o gene para a subunidade catalítica alfa da DNA polimerase. Foi também analisada a amplificação génica dos quatro genes seleccionados na forma promastigota do parasita (*in vitro*). Promastigotas de *L. infantum* foram tornados resistentes a antimoniato de meglumina, miltefosina, TFL-A3 e TFL-A6, e o número de genes quantificado por PCR em tempo real.

Foi demonstrado que a actividade anti-*Leishmania* dos compostos TFL-A6 e orizalina é bastante promissora, sendo muito semelhante à actividade do estibogluconato de sódio. TFL-A3 apresentou actividade anti-*Leishmania* mais reduzida. Os parasitas que *in vivo* sobreviveram ao tratamento com estibogluconato de sódio apresentaram aumento significativo do número de cópias dos genes *MDR1* e *PTR1*, sugerindo amplificação da capacidade de expulsão deste fármaco do interior do parasita e alterações na via metabólica do folato, reduzindo a quantidade de intermediários de espécies reactivas de oxigénio e azoto. Apesar de existirem duvidas devido ao facto das amostras serem provenientes de doentes que poderiam ter estado sujeitos a fármacos adicionais, a amplificação de *MDR1* tinha sido demonstrada em estudos anteriores. O presente estudo vem mostrar que a amplificação de *MDR1* ocorre de facto em parasitas que estiveram unicamente em contacto com estibogluconato de sódio. Os parasitas provenientes de murganhos tratados com TFL-A3 apenas apresentaram aumento do número de cópias do gene *MDR1*. Os parasitas isolados a partir do grupo tratado com TFL-A6 apresentaram amplificação dos genes *MDR1*, *MRPA* e *PTR1*. Esta amplificação indica que o mecanismo de resistência está associado ao efluxo do fármaco pelo parasita, ao sequestro do fármaco para compartimentos intracelulares do parasita e a alterações na quantidade de espécies reactivas de oxigénio e azoto que se formam. Nos parasitas que estiveram em contacto com a orizalina os genes *GSH1*, *MDR1* e *PTR1* apresentam aumento do número de cópias. No caso da orizalina o mecanismo usado parece estar relacionado com o aumento de efluxo do composto, alterações na quantidade de espécies reactivas de oxigénio e azoto e alterações no balanço do potencial reductor no interior do parasita. Nas experiências *in vitro* os promastigotas resistentes a antimoniato de meglumina, miltefosina, TFL-A3 ou TFL-A6 demonstraram resultados distintos dos encontrados nos parasitas resultantes dos murganhos que foram sujeitos a tratamento. Estas diferenças podem ser explicadas pelo modo como diferentes formas morfológicas do parasita reagem quando contactam com o fármaco. Nas experiências *in vivo* a forma amastigota encontrava-se internalizada pelos macrófagos do hospedeiro, retida no interior dos fagolisossomas, enquanto nas experiências *in vitro* a forma promastigota encontrava-se livre no meio de cultura. Os diferentes ambientes (amastigota – intracelular, rodeado por duas membranas, pH ácido, 37°C, sujeito a acções do sistema imunitário do hospedeiro; promastigota – livre, meio de cultura, pH neutro, 24°C) induzem a diferenciação pelo parasita de diferentes mecanismos de adaptação e sobrevivência. Os promastigotas resistentes a antimoniato de meglumina demonstraram um aumento significativo do número de cópias de *GSH1* e redução do número de cópias de *MDR1*. Os parasitas resistentes a miltefosina não apresentaram qualquer alteração significativa no número dos genes estudados. Os

parasitas resistentes a TFL-A3 apresentaram amplificação do gene *GSH1* e os resistentes a TFL-A6 demonstraram um aumento significativo do número dos genes *GSH1*, *MDR1* e *PTR1*.

Com este estudo é possível concluir que uma elevada actividade anti-*Leishmania* e uma baixa toxicidade pode não ser suficiente para um composto ser considerado uma boa alternativa aos fármacos actualmente usados. Resistência a TFL-A6 parece dever-se a indução de mecanismos semelhantes aos responsáveis pela resistência aos fármacos actualmente em uso. O desenvolvimento de menor sensibilidade ou resistência pode conduzir à rápida diminuição da eficiência anti-*Leishmania* deste composto ou mesmo à ineficácia no combate a estirpes já resistentes aos fármacos em uso. Todos estes factores devem ser tidos em consideração no desenvolvimento de novos compostos anti-*Leishmania*, prevenindo o aparecimento de estirpes menos susceptíveis mas também como forma de minimizar o investimento em fármacos que à partida não deveriam ser considerados como alternativa para tratamento da leishmaniose.

**Palavras-chave:** Leishmaniose, *Leishmania infantum*, resistência, novos fármacos, amplificação génica.

## ABSTRACT

Leishmaniasis, caused by intracellular protozoa of the genus *Leishmania*, is considered one of the most important human parasitic diseases in the world. *Leishmania infantum* is the agent responsible for zoonotic visceral leishmaniasis, the most severe and fatal form of the disease, in the Mediterranean region, including Portugal. Recently, the variation of parasite sensitivity to several drugs became a problem in some endemic areas for *Leishmania*. It was observed that the ability of *Leishmania* to selectively increase the number of gene copies is the main adaptation of these parasites to drug pressure, therefore responsible for resistance. Furthermore, all the currently used antileishmanial drugs do not conjugate high efficiency, low toxicity and an affordable cost. Therefore the development of new therapeutic compounds for leishmaniasis still is a priority. The potential of trifluralin (TFL) for the treatment of leishmaniasis was recently demonstrated and two analogues, TFL-A3 and TFL-A6, which presented higher antileishmanial activity and less cytotoxicity than TFL were analyzed in the present study. Another drug, oryzalin (ORZ), and conventional antileishmanial drugs (sodium stibogluconate, meglumine antimoniate and miltefosine) were also assessed. *In vivo* and *in vitro* studies were performed to confirm their antileishmanial activity and to access their potential to induce drug resistance in *L. infantum*. The genes for ABC-thiol transporter (MRPA), P-glycoprotein (MDR1), the enzyme gamma-glutamylcysteine synthase (GSH1) and pteridine reductase 1 (PTR1), already associated with drug resistance in previous studies, were selected and quantified by real-time PCR. The results revealed that TFL-A6 and ORZ have an antileishmanial potential similar to the conventional antileishmanial drugs. However, it was demonstrated that a relatively short period of treatment (10 days) is enough to induce significant gene amplification in the parasites that survived *in vivo* to the treatment. *In vitro* experiments demonstrated that the level of gene amplification in the promastigote form is different than in the intracellular amastigote form of the parasite when exposed to the same drugs. This study accentuates the need for understanding the mechanisms and evaluate the appearance of resistances when designing and investigating new antileishmanial drugs.

**Keywords:** Leishmaniasis, *Leishmania infantum*, drug resistance, new drugs, gene amplification.

## **I. INTRODUCTION**

### **1. Leishmaniasis**

#### **1.1. Epidemiology**

Leishmaniasis is a parasitic disease caused by intracellular protozoa belonging to the family *Trypanosomatidae* Doflein, 1901 and the genus *Leishmania* Ross, 1903 [Shaw, 1994; Croft *et al.*, 2006]. More than 20 species of *Leishmania* were identified as responsible for the disease in humans [Schallig & Oskam, 2002]. The parasite is transmitted to the vertebrate host by sandflies of the genus *Phlebotomus* Rondani & Berté, 1843 in the Old World and *Lutzomyia* França, 1924 in the New World [Desjeux, 2001].

*Leishmania* infections are in most cases zoonosis from wild or domestic mammals, and accidentally the parasite infects humans, although in East Africa and Indian subcontinent anthroponotic form of the disease can be found. The main domestic animal reservoirs are the dogs, while the sylvatic ones are small rodents and larger mammals, like foxes, wolves and jackals.

##### **1.1.1. In humans**

Leishmaniasis is considered one of the most important human parasitic diseases in the world, both in mortality and morbidity. According to the World Health Organization (WHO) there is an estimate of 2 million new cases every year, 350 million people are at risk of being infected, around 12 million people are already infected and 60 000 die every year. Increase of incidence and severity of this disease is due to infected human and dog migration, global warming altering the distribution of the vector, co-infection with immunosuppressive diseases and poverty [Desjeux, 2001; Neuber, 2008; Alvar *et al.*, 2012].

Leishmaniasis different clinical manifestations can be classified into three types: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL).

Cutaneous leishmaniasis is the most common form of the disease. The clinical manifestations can go from a single self-healing skin lesion to multiple localized or diffused lesions leading to a chronic state [Sádlová, 1999]. The clinical outcome depends mostly on the species of the parasite, but it is also influenced by the species of the vector



and the innate or acquired resistance of the human host [Herwaldt, 1999]. Although the majority of the cases lead to spontaneous healing and the skin lesions disappear in less than a year, around 15% of the cases lead to relapses and 2 to 40% of the cases of CL evolve into the mucocutaneous form of the disease. The species responsible for this form of leishmaniasis are: *Leishmania major*, *L. tropica*, *L. aethiopica*, *L. infantum*, *L. shawi*, *L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. peruviana*, *L. panamensis*, *L. guyanensis*, *L. lainsoni* and *L. naiffi* [Murray *et al.*, 2005; Neuber, 2008].

Mucocutaneous leishmaniasis consists in a progression of the cutaneous form, it happens when the infection reaches the mucosal regions. Can affect and destroy the tissues of the oronasal and pharyngeal cavities, and can lead to severe and painful facial mutilations. This form can happen as soon as one week after the appearance of the first skin lesion as well as many years after the cutaneous manifestations, showing that the parasite persists in the tissues for a long time. The species responsible for this form of leishmaniasis are: *L. braziliensis*, *L. panamensis* and *L. guyanensis*. There are also reports of mucocutaneous leishmaniasis caused by *L. donovani*, *L. major* and *L. infantum* in individuals with immunosuppressive diseases [Desjeux, 1996].

Visceral leishmaniasis is the most severe and fatal form of the disease, comprising a broad range of clinical manifestations. The parasite invades and multiplies in the organs of mononuclear phagocyte system such as the spleen, liver and lymph nodes and the symptoms are characterized by prolonged and irregular fever, splenomegaly, lymphadenopathy, hepatomegaly, pancytopenia, progressive anemia, weight loss and hypergamma-globulinemia with hypoalbuminemia. In many cases the infection does not take an acute or chronic course, remaining asymptomatic or subclinical and can even lead to a self-healing scenario [Sahni, 2012]. The species responsible for this form of leishmaniasis are: *L. donovani* and *L. infantum* [Berman, 1997].

### 1.1.2. In dogs

Canine leishmaniasis (CanL) is a chronic and systemic disease with a broad spectrum of non-specific clinical manifestations caused by *L. infantum* in domestic dogs (*Canis lupus familiaris*). The most frequent manifestations are skin lesions, however, the animals can show other clinical signs unrelated to cutaneous lesions as their main symptoms such as renal disease or any other symptoms characteristic of human VL. Since infection with the parasite does not always mean clinical illness there is a high prevalence of subclinical and asymptomatic infected animals in the endemic regions. In addition to the fact that the domestic dogs share the same habitat, frequently contact

and often travel with humans, this makes dog the most important reservoir for *L. infantum*. Other species of *Leishmania* were also found infecting dogs (*L. braziliensis*, *L. panamensis* and *L. peruviana*) and even though this species do not cause disease in these animals it may be that dogs act as reservoirs for this species as well [Reithinger *et al.*, 2003; Solano-Gallego *et al.*, 2011; Palatnik-de-Sousa, 2012].

### **1.1.3. Geographic distribution**

*Leishmania* occurs mostly in tropical and sub-tropical areas and is present in all continents, except Antarctica. Until the last decade, it was thought that Oceania and Southeast Asia were *Leishmania* free but recent findings prove otherwise [Rose *et al.*, 2004; Conlan *et al.*, 2011]. Endemic regions can be found in South and Central America, North and East Africa, Middle-East, Indian sub-continent, Central and Eastern Asia and South Europe, putting together a total of 98 countries considered endemic for *Leishmania*. More than 90% of all VL cases occur in just six countries: India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia. The prevalence of CL is more widely spread, with 70 to 75% occurring in ten countries around the globe: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru [Alvar *et al.*, 2012].

In Portugal as in the Mediterranean Basin countries, the species responsible for the majority of leishmaniasis cases is *L. infantum*, both in humans and animals. There are reports of leishmaniasis across the entire continental territory of Portugal, but three areas stand out as the most important endemic regions: Metropolitan area of Lisbon, Alto Douro and Algarve. The prevalence of CanL can go up to 20% in these endemic areas and between 2000 and 2009 there were 173 new cases reported of VL, mostly in children and immunocompromised individuals [Santos-Gomes *et al.*, 1998; Campino & Maia, 2010].

## **1.2. Life cycle**

*Leishmania* is a dimorphic parasite: promastigote form (Figure 1) occurs in the invertebrate host (sandfly) and the vertebrate host has the amastigote form (Figure 2). The promastigote, found in the midgut of the insect vector, has an elongated shape and a well-developed anterior flagellum. It is possible to characterize two stages of promastigote development: the procyclic stage and the metacyclic stage. Procyclic

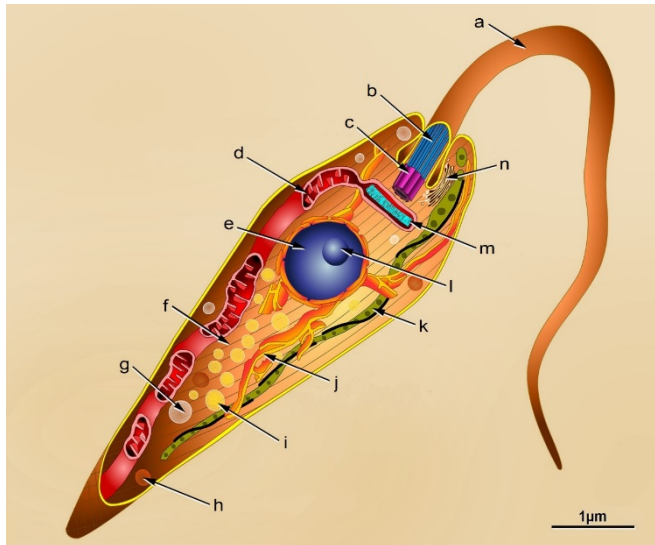


Figure 1. Schematic representation of a *Leishmania* promastigote. a – flagellum; b – axoneme; c – basal corpuscle; d – mitochondria; e – nucleus; f – subpellicular microtubules; g – glycosome; h – lipid inclusions; i – acidocalcisome; j – endoplasmic reticulum; k – multivesicular tubule; l – nucleolus; m – kinetoplast; n – Golgi complex. Adapted from Teixeira *et al.* (2013).

promastigotes are not infectious, are in continuous division and have a shorter flagellum. On the other hand, metacyclic promastigotes are highly infectious, are long and narrow and have a large flagellum [Killick-Kendrick, 1990; Ashford, 2000]. Amastigotes have a round shape and no external flagellum. Being an obligatory intracellular form it can only survive and multiply inside parasitophorous vacuoles of phagocytic cells.

Parasites are transmitted to the vertebrate host when the infected female sandfly takes a blood meal, inoculating the promastigotes in the vertebrate's dermis. Parasites are then phagocytized by macrophages and once inside the cell they transform into amastigotes. Amastigotes multiply inside phagocytic cells that are later ingested by another sandfly when it takes a blood meal. Ingested cells are digested in the sandfly gut and the free amastigotes reach the midgut where they transform into promastigotes. Promastigotes multiply and later migrate into the proboscis of the sandfly, from where they will be introduced into the vertebrate host dermis when a blood meal occurs [Cohen-Freue *et al.*, 2007; Dostálová & Volf, 2012] (Figure 3).

### 1.3. Parasite/vertebrate host interaction

In the vertebrate, destruction of invading microorganisms is essentially executed by two types of

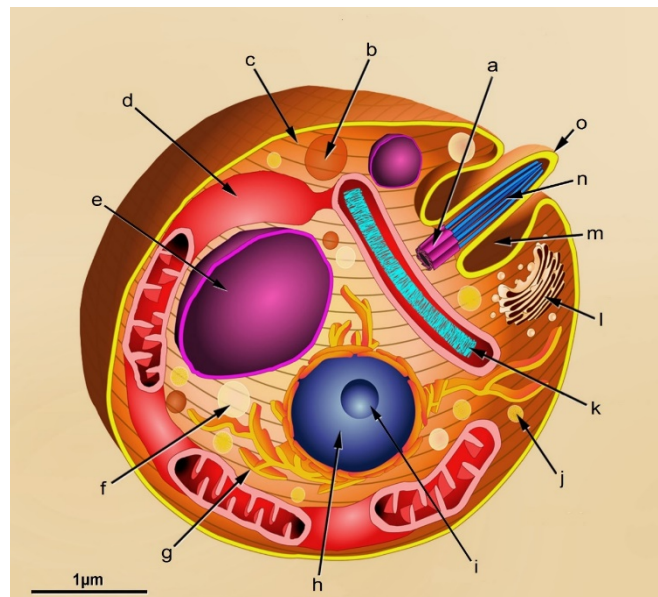
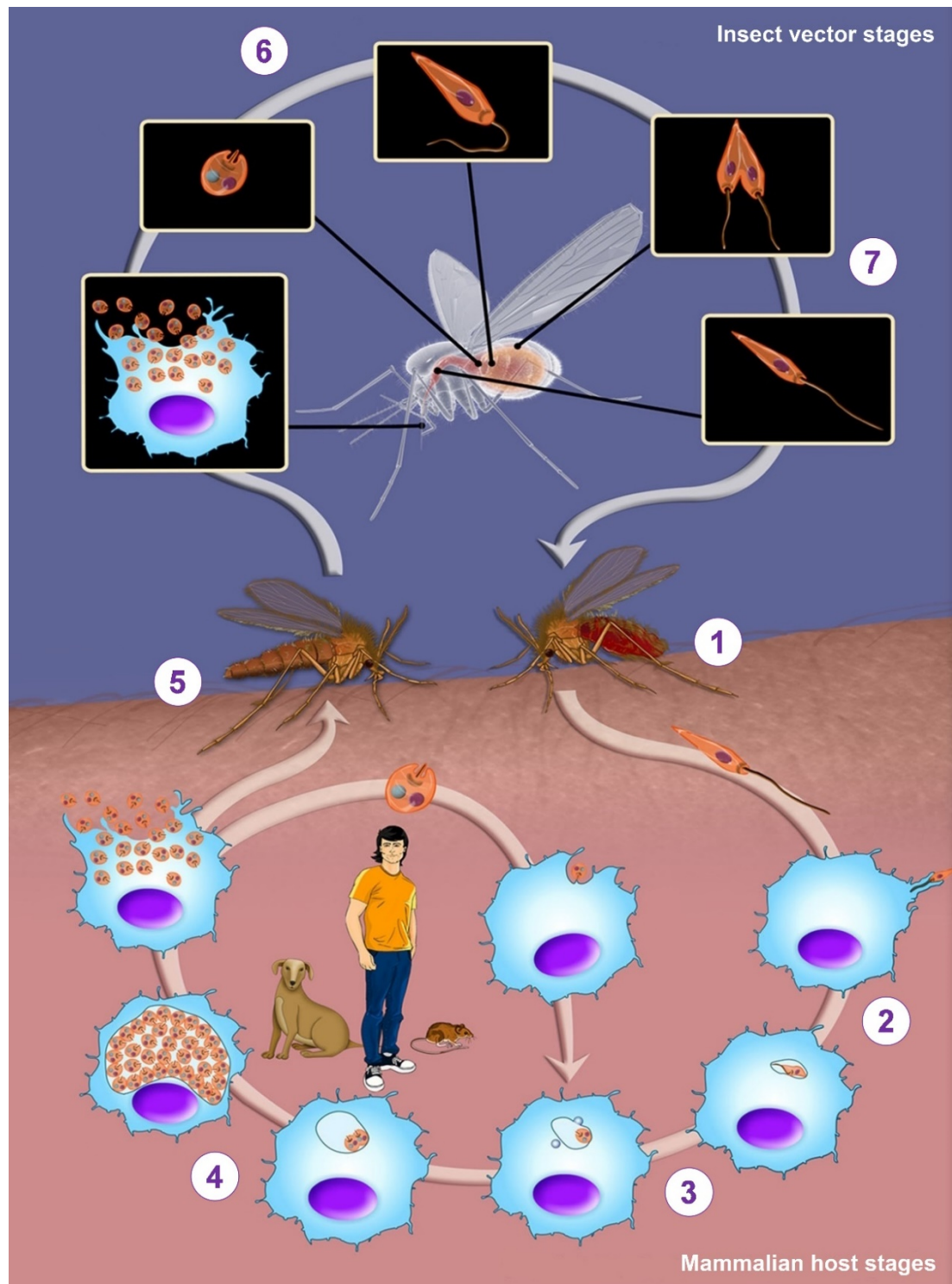


Figure 2. Schematic representation of a *Leishmania* amastigote. a – basal corpuscle; b – lipid inclusions; c – subpellicular microtubules; d – mitochondria; e – megosome; f – glycosome; g – endoplasmic reticulum; h – nucleus; i – nucleolus; j – acidocalcisome; k – kinetoplast; l – Golgi complex; m – flagellar pocket; n – axoneme; o – flagellum. Adapted from Teixeira *et al.* (2013).



**Figure 3.** Life cycle of *Leishmania* in the mammalian host and insect vector. 1 – Female sandfly takes a blood meal and inoculates the promastigotes; 2 – Promastigotes are phagocytized by macrophages; 3 – Transformation into amastigotes inside the parasitophorous vacuole; 4 – Amastigote replication; 5 – Ingestion of infected macrophages or free amastigotes by the female sandfly when taking a blood meal; 6 – Transformation into promastigotes in the sandfly midgut; 7 – Promastigotes replication and transformation into metacyclic promastigotes. Adapted from Teixeira *et al.* (2013).

cells, the macrophages and the neutrophils. When inoculated in the vertebrate dermis, *Leishmania* promastigotes are phagocytized by macrophages (Figure 4). Within these cells, the parasites stay inside parasitophorous vacuoles that fuse with lysosomes

containing proteolytic enzymes, forming phagolysosomes [Bogdan & Röllinghoff, 1998]. Within phagolysosomes production of reactive oxygen and reactive nitrogen molecules occur in order to destroy the invader organism [Slauch, 2011].

*Leishmania* has the ability to slow down the fusion of these vacuoles assuring the successful transformation into amastigotes. Amastigotes present high enzymatic activity and using catalase, superoxide dismutase and glutathione peroxidase are able to eliminate the products of

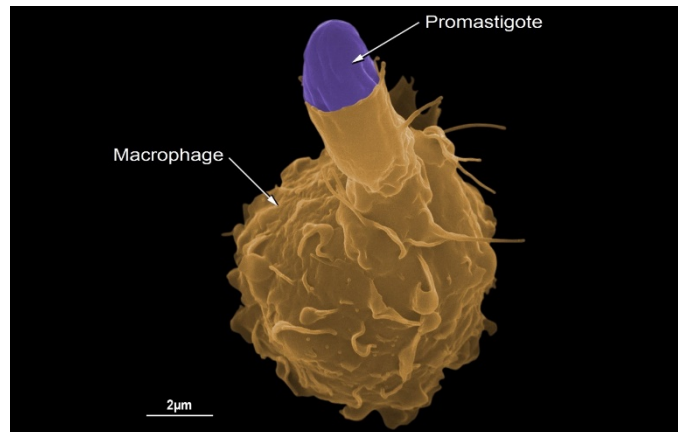


Figure 4. Scanning electron microscopy micrograph of a *Leishmania* promastigote being phagocytized by a macrophage. Adapted from Teixeira *et al.* (2013).

oxidative burst [Meshnick & Eaton, 1981; Murray, 1981]. The lipophosphoglycan of *Leishmania* is also a potent inhibitor of the proteins responsible for activation of oxidative burst as well as capable of interfering with the production of nitric oxide inside the phagolysosomes [Bogdan & Röllinghoff, 1998].

### 1.4. Diagnostic

Because of the wide spectrum of clinical manifestations this disease represents a diagnostic challenge, requiring confirmatory tests to decide if patients should be treated. These tests require a high sensitivity and specificity due to the leishmaniasis clinical severity and to the fact that drugs currently used for the treatment have high toxicity and can be very expensive. Microscopy still is the standard technique for leishmaniasis diagnosis in endemic areas [Reithinger & Dujardin, 2007]. Giemsa-stained lesion biopsy smears or aspirates from the lymph node, bone marrow or spleen are used with high specificity to identify the presence of the parasite. Laboratory cultures of the biopsies and aspirates are also used to diagnose leishmaniasis. However, microscopy and culture diagnosis tend to have low sensitivity and be highly variable [Herwaldt, 1999]. Several immunological techniques are also used as complementary to direct parasitological detection. Tests based on indirect fluorescence antibody, enzyme-linked immunosorbent assay and western blot have shown high diagnostic accuracy. Two serological tests were specifically developed for VL, the direct agglutination test and the rK39-based immunochromatographic test. Both tests showed values higher than 90% for both sensitivity and specificity [Chappuis *et al.*, 2007]. One test was developed for CL, the

Montenegro skin test where an intradermal injection of dead promastigotes is given to the patient and examined 48 hours later to see if a delayed-type hypersensitivity has formed. This test has a very high sensitivity and specificity however does not distinguish between past and present infections. The most reliable approach in leishmaniasis diagnosis at this moment is the molecular approach based on polymerase chain reaction (PCR) assays. PCR has the highest sensitivity and specificity (up to 100%) of all the diagnostic methods for leishmaniasis, can diagnose independently of parasite species and have been shown to be better than direct parasitological test specially on samples with low parasite loads or samples from less intrusive sources [Reithinger & Dujardin, 2007].

### 1.5. Control

Control strategies focus on two major areas: vector control and reservoir control. For vector control the most widely strategy used is the spraying of households with insecticide and the use of insecticide treated curtains and bed nets. However this strategy was only proved to be effective in small cluster areas and the results cannot be extrapolated elsewhere [Davies *et al.*, 2003]. For reservoir control there are several strategies. The culling of dogs have been widely used in Brazil and China. But while in China the indiscriminate culling of dogs had positive results, recent studies showed that has not been effective in Brazil, where only serological positive dogs were euthanized. The use of insecticide treatment of dogs and insecticide dog collars has shown good results in controlling parasite transmission to humans. The treatment of reservoirs has also shown good results in reducing transmission [Piscopo & Azzopardi, 2006]. With the recent introduction of vaccines for CanL (in Europe and Brazil) a decrease in parasite transmission is expected, but is still too soon to reach conclusions.

### 1.6. Treatment

The first-line treatment for every type of leishmaniasis still remains the pentavalent antimonials ( $Sb^V$ ) in the forms of sodium stibogluconate (STB) and meglumine antimoniate (MGA). In many endemic areas these drugs have been used in the combat of *Leishmania* infection for more than 70 years. In the last two decades cheaper generic forms with equivalent results of the branded products emerged and its use was even more widespread. Despite having good efficacy these drugs are also toxic and

responsible for life-threatening adverse side effects, including cardiac arrhythmia and acute pancreatitis [Chappuis *et al.*, 2007]. STB (100 mg/ml of Sb<sup>V</sup>) and MGA (85 mg/ml of Sb<sup>V</sup>) are given intravenously and intramuscularly, usually at a dose of 20 mg of Sb<sup>V</sup>/kg/day for up to 28 days, with a recommended maximum dose of 850 mg/day. Shorter periods of treatment have been proposed with equal efficiency (minimum of 10 days) [Wortmann *et al.*, 2002] but this may increase the predisposition to drug-resistance even more [Piscopo & Azzopardi, 2006]. In some areas, especially in India, the failure rate for treatment with Sb<sup>V</sup> is more than 50% due to drug-resistance [Sundar, 2001], largely because of inappropriate use of antimony treatment. Because of the increasing drug-resistance and the adverse side-effects, research on new drugs was continued and lead to second-line drugs, amphotericin B (AmB) and miltefosine (MILT), most commonly used nowadays to fight resistant infections or the most severe cases of the disease.

AmB is mostly used in resistant cases of leishmaniasis where the Sb<sup>V</sup> treatment fails, but its high toxicity and prolonged period of treatment (given intravenously for up to 60 days) are a limiting factor despite the high cure rate (97%). An alternative is the liposomal form of AmB which is equally effective but less toxic. However it is still prohibitively expensive, especially in poor countries where the resistances to Sb<sup>V</sup> are more significant and this drug is needed most [Piscopo & Azzopardi, 2006]. In Southern Europe the use of this drug as a first line-treatment to replace Sb<sup>V</sup> have increased, possibly because the access to the drug is economically less restrictive and because of the growing resistance to Sb<sup>V</sup> augmented by the use of MGA to treat dogs with leishmaniasis [Gradoni *et al.*, 2003].

MILT is the first effective drug against *Leishmania* (94% cure rate) to be taken orally. MILT is a simple, very stable, relatively safe and highly efficient molecule with the capacity not only to directly kill *Leishmania* parasites but also to enhance both T cell and macrophage activation and the production of reactive nitrogen and oxygen intermediates by these cells [Soto *et al.*, 2007]. These properties and the potential to be distributed in resource-poor areas make this drug an excellent candidate for a generalized first-line drug. However concerns about compliance with treatment and resistance have already been expressed [Murray *et al.*, 2005].

Other compounds have been proven efficient antileishmanial drugs, particularly drugs directed for CL treatment. Pentamidine, paromycin, fluconazole and ketoconazole are examples of drugs already used in the treatment of CL, but as the more commonly used first-line drugs there are life-threatening adverse side effects and the potential for the emergence of drug-resistance. Drugs as allopurinol and imiquimod are also being used in the treatment of leishmaniasis in combination with Sb<sup>V</sup> in cases of drug resistance or very severe infections [Piscopo & Azzopardi, 2006].



## 2. Gene amplification in *Leishmania*

### 2.1. Gene amplification

When a slow accumulation of RNA and proteins is not enough for the normal functioning of the cells, multiple copies of genes can be found, allowing a faster expression than the one achieved from a single gene copy. Multiple gene copies can be found in most cells, although in some cases specific genes, or sets of genes, are amplified in specific types of cells. Developmentally regulated gene amplification is common. However, gene amplification not related to cell development has been described as an abnormal process found in both prokaryotes and eukaryotes. Gene amplification can be found within chromosomes or in extrachromosomal elements. Within chromosomes is found in homogeneously staining regions, following the same pattern of replication and segregation as the rest of the chromosome. The extrachromosomal elements can appear as either linear or circular amplicons, with direct (head-to-tail) or inverted (head-to-head) repeats. These amplicons usually lack centromeres and telomeres and in the absence of a selective pressure tend to be lost [Stark & Wahl, 1984]. Gene amplification has been studied and related to several adaptations, ranging from the transport of nutrients within the cells to adaptive modifications in domesticated animals, and also plays a major role in adaptation to antibiotics and drugs in several types of cells. This adaptation to drugs via gene amplification was reported and widely studied in mammal tumor cells [Kondrashov, 2012].

*Leishmania infantum*, like other species of *Leishmania*, have a small genome (approximately 32100000 bp) with a small amount of genes (8241 coding genes), providing an easy target for gene amplification studies [Rogers *et al.*, 2011]. Also, the unusual lack of transcriptional control of *Leishmania*, where expression is regulated almost exclusively by post-transcriptional mechanisms [Martínez-Calvillo *et al.*, 2003; Martínez-Calvillo *et al.*, 2010] is an advantage for success of gene amplification. Studies have shown that amplified DNA can reach as much as 10% of the total DNA in *Leishmania*, as extrachromosomal circular or linear amplicons. Subchromosomal amplifications were also reported in *Leishmania*, consisting of relatively small chromosomes (250000 bp or less) occurring in multiple copies (up to 40 per cell) and only present in some but not all isolates from numerous *Leishmania* species. Initially thought that this was the result of some kind of horizontal transmission, current data suggest that is the result of extrachromosomal amplification where the amplicons acquired at least one new telomere during their formation, presumably by the action of



leishmanial telomerase. The greatest number of copies of these amplicons found in some isolates suggests evasion of the mechanisms that normally limit the number of chromosomes. Since no biological role for this subchromosomal amplicons have been demonstrated so far it is believed that they may provide subtle growth advantages, particularly during adaptation to *in vitro* culture [Beverley, 1991].

## 2.2. Circular amplicons

Opposite to tumor cells where there is little or no need of homologous sequences for the rearrangement of chromosomal sequences to produce circular amplicons, in *Leishmania* homologous sequences are needed for the generation of amplicons [Grondin *et al.*, 1996]. Also, unlike mammalian amplifications, *Leishmania* amplifications appear to be homogenous and possess the minimum number of DNA rearrangements necessary to generate an amplicon from the chromosomal gene; one for the direct amplification (Figure 5a) and two for the inverted amplification (Figure 5b). They usually range from 30000 bp to 200000 bp.

Secondary rearrangements of amplified DNA were considered rare events in *Leishmania* but studies showed that gene amplification is more dynamic than anticipated. It is now suggested that gene rearrangements leading to gene amplification are widespread and that circular amplicons are generated from linear amplicons [Grondin *et al.*, 1998]. There are three types of amplifi-

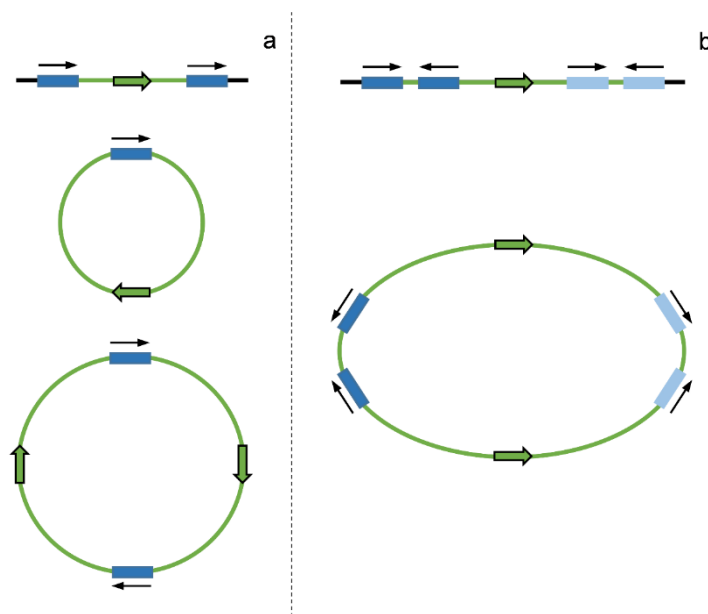


Figure 5. Schematic representation of DNA structures of direct amplification (a) and inverted amplification (b) in *Leishmania*. The blue boxes represent repetitive DNA sequences whose orientations are indicated by black arrows. Green arrows indicate the orientation of the gene being amplified (green line).

cation described in *Leishmania*. Deletion amplification (Figure 6a), where the copy of wild-type chromosomal locus is deleted during the generation of the extrachromosomal amplicon, resulting in a heterozygous deletion line. Conservative amplification (Figure 6b), where the generation of the amplicon produces no alterations in chromosomal structure or ploidy. And, duplicative amplification (Figure 6c), where several additional

copies are found inserted into the locus in addition to the amplified amplicon. As stated before, this extrachromosomal amplicons can be either a result of direct amplification or inverted amplification [Beverley, 1991].

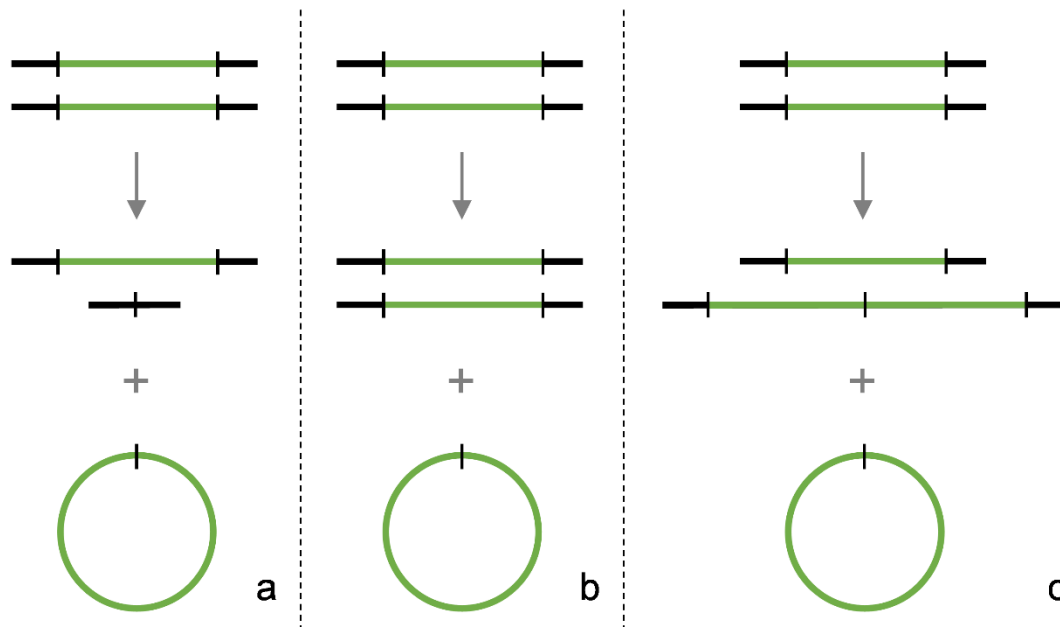


Figure 6. Schematic representation of deletion (a), conservative (b) and duplicative (c) amplification. The black lines represent flanking chromosomal DNA, the green lines represent DNA segments that give rise to extrachromosomal circular amplicons in addition to being either deleted, conserved or duplicated in the chromosome.

### 2.3. Mechanism

*Leishmania* amplicons, whose structure have been characterized, seem to rule out gene amplification models that imply alteration of the parental chromosome structure (like sister chromatid exchange or recombination in the absence of re-replication). Extrachromosomal amplicons are likely to be generated from extra copies of the chromosomal locus formed by re-replication during the cell cycle. The onionskin model (Figure 7) seems to explain the initial structure of the re-replication. This hypothetical structure has this name because it resembles the skin layers of an onion when multiple rounds of replication initiate in the same origin within a single replication bubble in a given replication cycle. The flexibility of this model allows to virtually

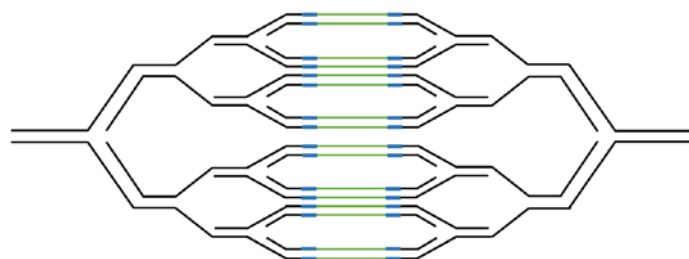


Figure 7. Schematic representation of the onionskin model. Overreplication gives rise to a layer structure. The black lines represent genomic DNA and the blue areas correspond to repetitive DNA sequences flanking the DNA fragments (green) that will be amplified.

obtain all types of amplification. Unaltered DNA strands can maintain the normal chromosome structure after the collapse of the onionskin, and the copies generated are released as extrachromosomal amplicons, explaining this way the conservative amplification. Rearrangements in the chromosome strand can lead to deletion plus amplicon formation, explaining the deletion amplification. Rearrangements between the chromosomal strand and the replicated segments can lead to duplicative amplification within the chromosome, with the release of the extra copies generated as extrachromosomal amplicons. It also explains the generation of linear amplicons whenever the released copies acquire telomeres by the action of a leishmanial telomerase. Since the formation of amplicons in *Leishmania* is associated with homologous repetitive sequences it is supposed that these sequences give rise to hotspots for DNA rearrangement and amplification. Genes flanked by direct repeats undergo direct amplification (Figure 5a) while genes flanked by inverted repeats undergo inverted amplification (Figure 5b). The final step of the mechanism consists in the ability to increase and maintain the copy number of the circular amplicons. A selective pressure can ensure that a sufficient number of copies of a specific gene are maintained in the parasites. However, little is known about the mechanism for autonomous replication of the amplicons, only that no specific sequences seem to be required for replication [Beverley, 1991; Papadopoulou *et al.*, 1994].

## **2.4. Response to drug pressure**

*Leishmania* differs from other closely related protozoan parasites in the reaction to drug pressure. In *Trypanosoma brucei* gene amplification is extremely rare and in parasites of genus *Plasmodium* the gene amplification is mainly chromosomal, therefore in these parasites the increase of gene copy number is very reduced, so other mechanisms of overexpression of multidrug resistance genes play an important role. On the other hand, it was observed that the ability of *Leishmania* to selectively increase the number of gene copies is responsible for resistance. This was possible due to the relatively low genomic complexity of *Leishmania*. Gene amplification, mostly as extrachromosomal amplicons, is now considered the main adaptation response of these parasites to drug pressure [Beverley, 1991; Papadopoulou *et al.*, 1998]. Changes in membrane permeability, allowing a reduction of intracellular drug accumulation, or a decrease in drug intake and inactivation of the drug by metabolism or sequestration are the mechanisms used by *Leishmania* to survive when in contact with antileishmanial compounds. The increase of gene copy number will not only directly increase the level

of expression but also augment the probability of point mutations to appear, which may confer advantage to the parasite when in contact with antileishmanial drugs [Croft *et al.*, 2006].

### 3. Drug resistance in *Leishmania*

#### 3.1. Pentavalent antimonials

Pentavalent antimonials ( $\text{Sb}^{\text{V}}$ ) are considered pro-drugs. In order to acquire antileishmanial activity these compounds require biological reduction to the trivalent form ( $\text{Sb}^{\text{III}}$ ). Although the site of reduction remains unclear data suggests that it happens inside the parasite as well as within the macrophage. The pentavalent form is known to stimulate the infected macrophages, increasing the oxidative/nitrosative stress on the intracellular parasites.  $\text{Sb}^{\text{III}}$  acts directly on the parasite, deregulating its redox-balance.  $\text{Sb}^{\text{V}}$  is taken up by the parasite by an unidentified transporter and  $\text{Sb}^{\text{III}}$  enters via aquaglyceroporin (AQP1). Reduction of  $\text{Sb}^{\text{V}}$  to  $\text{Sb}^{\text{III}}$  inside the parasite can be a thiol dependent non-enzymatical spontaneous reduction or enzymatically catalyzed by thiol-dependent reductase 1 (TDR1) or arsenate reductase 2 (ACR2). Maintenance of redox balance inside the parasite is performed by trypanothione ( $\text{T}(\text{SH})_2$ ) and trypanothione reductase (TR) which reduces trypanothione disulfides ( $\text{TS}_2$ ) to  $\text{T}(\text{SH})_2$ , keeping the redox potential low.  $\text{T}(\text{SH})_2$  is synthesized by condensation of glutathione and spermidine, where gamma-glutamylcysteine synthase (GSH1) is the key enzyme for glutathione (GSH) synthesis.  $\text{Sb}^{\text{III}}$  inhibits TR and can form conjugates with  $\text{T}(\text{SH})_2$  and glutathione, leading to an increase in redox potential (Figure 8) [Wyllie *et al.*, 2004; Decuypere *et al.*, 2012].

In order to acquire resistance to antimonials it was shown that *Leishmania* can use different strategies. A decrease in the reduction of  $\text{Sb}^{\text{V}}$  to  $\text{Sb}^{\text{III}}$ , by reducing the levels of TDR1 and ACR2 inside the parasite leads to increase resistance. However, it is not effective in protecting from external  $\text{Sb}^{\text{III}}$ . In strains exhibiting decreased susceptibility to  $\text{Sb}^{\text{III}}$  a decrease in AQP1 expression was noted, although no difference in gene copy number was found. Decreasing the influx of  $\text{Sb}^{\text{III}}$  is probably the first barrier of the parasite to counteract the action of antimonials. An increase in the synthesis of glutathione and trypanothione from cysteine was also reported in resistant strains. This helps to restore thiol redox potential perturbed by the accumulation of  $\text{TS}_2$ , caused by the inhibition of TR activity by  $\text{Sb}^{\text{III}}$ . It also increases the spontaneous formation of  $\text{Sb}^{\text{III}}$  conjugates with either glutathione,  $\text{T}(\text{SH})_2$  or both.  $\text{Sb}^{\text{III}}$ /thiols conjugates can be

sequestered by ABC-thiol transporter MRPA (MRPA) into intracellular organelles or pumped out by an uncharacterized transporter (Figure 8). Previous studies also shown that an increase in MRPA alone cannot confer resistance to  $Sb^{III}$  in *Leishmania* and other

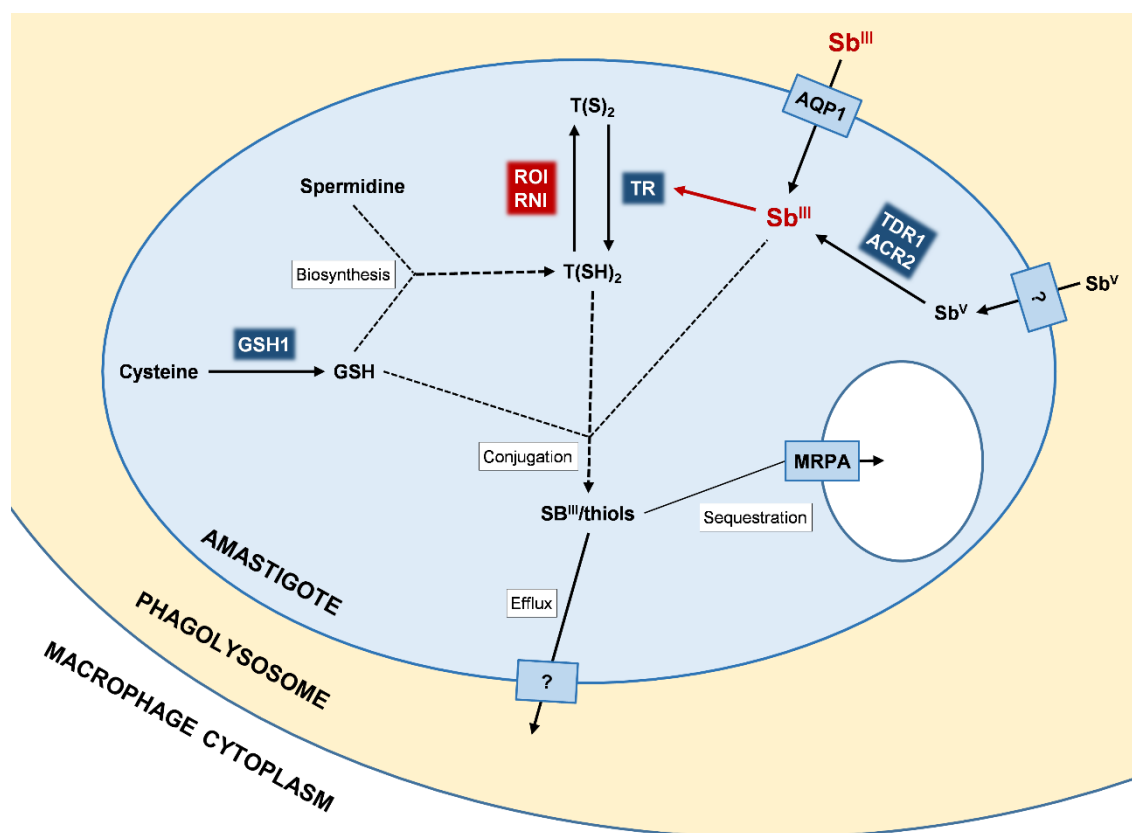


Figure 8. Overview of *Leishmania* pathways involved in response and resistance to pentavalent antimonials ( $Sb^V$ ). ACR2 – Arsenate reductase 2; AQP1 – aquaglyceroporin; GSH – Glutathione; GSH1 – Gamma-glutamylcysteine synthase; MRPA – ABC-thiol transporter MRPA; ROI – Reactive oxygen intermediates; RNI – Reactive nitrogen intermediates;  $Sb^{III}$  – Trivalent antimonials;  $Sb^{III}/thiols$  – Conjugates of  $Sb^{III}$  with  $T(SH)_2$  and/or GSH; TDR1 – Thiol-dependent reductase 1; TR – Trypanothione reductase;  $T(SH)_2$  – Trypanothione;  $T(S)_2$  – Trypanothione disulfide.

factors are needed. GSH1 seems to play a major role in the resistance conferred by MRPA, and although an increase of GSH1 substantially increases the resistance when conjugated with an increase of MRPA, it was shown that normal levels of GSH1 are enough for the MRPA-related resistance to occur [Callahan *et al.*, 1994; Grondin *et al.*, 1997; Haimeur *et al.*, 2000; Croft *et al.*, 2006; Ashutosh *et al.*, 2007; Jeddi *et al.*, 2011].

### 3.2. Miltefosine

Miltefosine (MILT) also known as hexadecylphosphocholine was only recently introduced as an antileishmanial drug but even before this introduction concerns about the emergence of resistance to MILT were already present. Data from a phase IV

treatment trial show a relapse rate twice as high when compared to first-line treatment. The multitude of proposed mechanisms of action for MILT and the contradictory data from several studies may indicate that more than one molecular site of action is used. These mechanisms seem to be related to alterations in the lipid metabolism, mediation of apoptosis-like cell death, mitochondrial dysfunction and immunomodulation. A perturbation of ether-phospholipid metabolism related to inhibition of glycosomal alkyl-specific acyl-CoA acyltransferase was found in *Leishmania* when treated with MILT. Also, a decrease in phosphatidylcholine and an increase in phosphatidylethanolamine was observed. This suggests that MILT is responsible for the alteration of the composition of the parasite membrane. Apoptosis-like cell death was also reported in parasites exposed to MILT. This usually happens after exposure to reactive oxygen species (ROS) and lead to DNA fragmentation, nuclear condensation, loss of cell volume and consequently cell death. Therefore, MILT may be responsible for disrupting the intracellular redox balance of the parasite that allows it to tolerate the ROS inside the phagolysosome. The involvement of mitochondrial dysfunction was also investigated and the inhibition of cytochrome-c oxidase was observed. This inhibition was responsible for a substantial reduction in the mitochondrial membrane potential. As an additional contributory factor MILT was shown to have immunomodulatory proprieties, being able to enhance interferon-gamma receptors in infected macrophages and thereby increase T helper cell type 1 response, necessary to fight the parasite infection [Croft *et al.*, 2006; Soto *et al.*, 2007; Dorlo *et al.*, 2012].

Emergence of drug resistance to MILT *in vivo* has not yet been described, although a strain of *L. infantum* with decreased susceptibility was recently isolated from a non-responsive HIV/VL patient. *In vitro* studies demonstrated that it is easy to induce resistance to MILT in *Leishmania*. These studies concluded that a defect in drug internalization and increased drug efflux from the parasite were the possible mechanisms of resistance. Two *Leishmania* lipid translocases, putative miltefosine transporter protein (MT) and MT non-catalytic subunit protein ROS3 play an important role in maintaining the phospholipid asymmetry of the parasite membrane. Their low expression was proven to be directly related to resistance to MILT, since a lower amount of these proteins leads to a decrease in the internalization of the drug into the parasite. Overexpression of P-glycoprotein MDR1 (MDR1) and ABC subfamily G-like transporters ABCG6 and ABCG4 was also implicated in MILT resistance, being responsible for a decrease in drug accumulation in the parasite. Several single point mutations in the genes coding these proteins were also observed *in vitro* [Coelho *et al.*, 2012; Dorlo *et al.*, 2012; Luque-Ortega *et al.*, 2012].

### 3.3. Amphotericin B

Amphotericin B (AmB) is a polyene antibiotic that binds preferentially to ergosterol, the major sterol of *Leishmania*. AmB binding to ergosterol causes the formation of transmembrane AmB channels which alter the permeability to cations, water and glucose and affect membrane-bound enzymes. These alterations eventually lead to the parasite death. Studies have also shown that AmB binds to the cholesterol present in the macrophages, decreasing the ability of the parasite to enter these cells and modulates macrophage activity by inducing the production of proinflammatory cytokines and ROS [Chia & McManus, 1990; Mozaffarian *et al.*, 1997].

Apart from relapses of VL patients co-infected with HIV, no other signs of resistance were found so far for AmB. These relapses were also proven not to be a cause of resistance of the parasite to AmB, thus all the data available arrive from *in vitro* experiments. AmB-resistant *Leishmania* showed significant changes in membrane sterol composition, where most of the ergosterol was replaced by a precursor, cholesta-5,7,24-trien-3 $\beta$ -ol. This was a result of a loss of function of sterol-methyltransferases responsible for ergosterol biosynthesis whose genes most likely suffered deletion amplification [Croft *et al.*, 2006; Paila *et al.*, 2010].

### 3.4. Strategies to combat drug resistance

There are several strategies to combat drug resistance that only now are starting to be taken into consideration, mostly because of the increase and spreading of resistance to Sb<sup>V</sup>.

The variation of parasite sensitivity to several drugs is a problem in areas endemic for more than one species of *Leishmania*. This can be solved by improvement of the diagnostic methods, especially non-invasive tests with high sensitivity and specificity for precocious detection of antileishmanial antigen and able to distinguish different species of *Leishmania*. A precocious, robust and accurate diagnosis would improve the treatment, allowing a correct choice of drug, the right dosage and determine the period of treatment. This would also aid to minimize the possible failure of treatment and consequently reduce the probability of the emergence of drug resistance.

The monitoring of the therapy also plays an important role. One of the causes for the appearance of drug-resistant *Leishmania* is believed to be the reduced level of compliance with treatment, since disease symptoms disappear before the full clinical cure. A better strategy in this field is needed, perhaps the implementation of control

programs like the ones already in practice by the World Health Organization for tuberculosis. These programs rely on the directly observed treatment, short-course (DOTS) strategy. The DOTS strategy combines government commitment, case detection, standardized treatment, direct observation of the patient and compliance with treatment by healthcare workers, regular drug supply and standardized recordings and, a reporting system that allows assessment of the treatment results.

The distribution and cost of the drugs are also another important factor. The elevated cost of the more efficient and safe drugs and their unavailability in several public health institutions (especially in countries of low income) is something that needs to be changed. Either by researching on new, highly effective and cheaper drugs or by implementation of programs by governments or private institutions able to distribute the right drugs among those that need them.

Another way to prevent the emergence of drug resistance is to monitor the susceptibility of the parasite strains to the common drugs. This could be done by create and implement a routine test in endemic areas with high risk of resistance emergence. This would allow a better choice of treatment and higher efficacy rate in the elimination of the disease. Also, the use of combination therapies would be facilitated. Given that the possibility of resistance development to a single therapeutic agent is high, the chance of developing simultaneous resistance to two compounds with different targets would lower the probability of resistance emergence by a great amount.

Furthermore, the most relevant strategy is probably the research of new drugs and new targets. An adequate amount of drugs with different targets and no cross-resistance is the most efficient way to avoid the appearance of drug resistance [Sundar, 2001; Sundar & Rai, 2002; Croft *et al.*, 2006; Jain, 2010; Antinori *et al.*, 2012].

### 3.5. New drugs

In spite of all the currently used antileishmanial drugs, none of them conjugates high efficiency, low toxicity and an affordable cost. So it is still a priority the development of new therapeutic compounds for leishmaniasis.

Current research on new antileishmanial drugs is especially focused on already existing compounds used for the treatment of other diseases, compounds from natural sources, like plants, and even formulations already in use as herbicides. Several compounds of diverse molecular structure with potential antileishmanial activity have been isolated. Among these compounds, the most notable are dinitroanilines, alkaloids, particularly indoles, naphthylisoquinolines, bisbenzylisoquinolines, benzoquinolizidines,



triterpenes, steroids, saponins, sesquiterpenes, diterpenes and flavonoids, mainly isoflavones and chalcones [Marques *et al.*, 2008; Passero *et al.*, 2013].

#### 4. Dinitroanilines derivatives

The potential of trifluralin (TFL, Figure 9a) for the treatment of leishmaniasis was recently demonstrated. TFL is a dinitroaniline, widely used in herbicidal formulations, that binds to the parasite tubulins (the main structural component of microtubules) inhibiting the growth and differentiation of the parasite. And, since it lacks affinity to animal tubulins, is not toxic to mammals. However, its use is limited by its low water solubility and easy sublimation. To overcome this, chemical modifications were introduced and several TFL analogues (TFL-A) were screened for better solubility and improved antileishmanial activity [Esteves *et al.*, 2010]. The analogues TFL-A3 (Figure 9b) and TFL-A6 (Figure 9c) presented high antileishmanial activity and low cytotoxicity. Oryzalin (ORZ, Figure 9d), another dinitroaniline also showed good *in vitro* antileishmanial activity and low cytotoxicity [Lopes *et al.*, 2012].

Mice previously infected with *L. infantum* and treated with TFL-A3, TFL-A6, ORZ or the conventional antileishmanial drug STB demonstrated that a complete elimination of *Leishmania* was not attained [Marques *et al.*, 2008], therefore it was theorized that the surviving parasites were less susceptible or resistant to the drug used in the treatment.

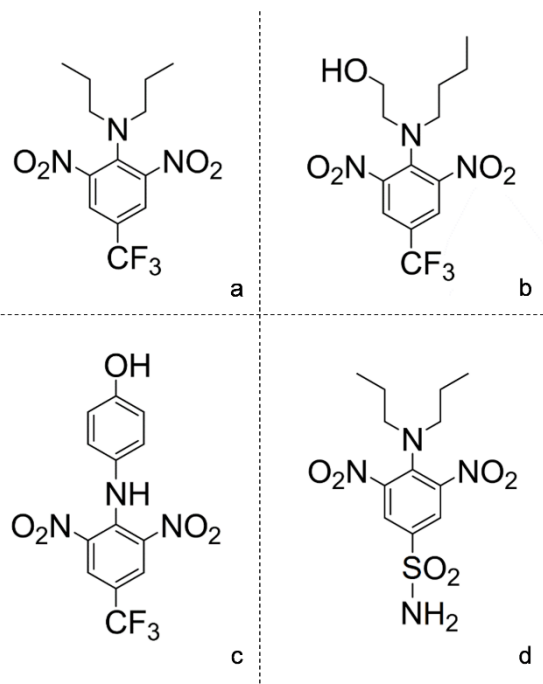


Figure 9. Chemical structure of TFL (a), TFL-A3 (b), TFL-A6 (c) and ORZ (d).

## II. OBJECTIVES

Since it was theorized that parasites that survived exposure to conventional and new antileishmanial drugs in animal models were less susceptible or resistant to the drug used in the treatment, the main objective of this study was to analyze the level of gene amplification of resistance-related genes in these *L. infantum* strains and evaluate the resistance achieved by the parasite to the different drugs used.

To evaluate the antileishmanial activity of a commonly used antileishmanial drug (STB) and new chemically synthesized compounds (ORZ, TFL-A3 and TFL-A6) and their potential to generate drug-resistant parasite strains, previously infected mice were treated with these compounds, and the parasites that survived to the treatment quantified and collected for DNA extraction and real-time PCR analysis.

Previous studies linked the amplification of four specific genes to drug resistance in clinical strains of *L. infantum*: the genes for energy depended transporters MRPA (*MRPA*, RefSeq XM\_001465669.1) and MDR1 (*MDR1*, RefSeq XM\_001468445.1), the gene for the enzyme GSH1 (*GSH1*, RefSeq XM\_001464941.1) involved in glutathione and trypanothione synthesis and, the gene for pteridine reductase 1 (*PTR1*, RefSeq XM\_001465671.1) an enzyme involved in the folate metabolic pathways [Mary *et al.*, 2010]. Therefore, these four genes (*MRPA*, *MDR1*, *GSH1* and *PTR1*) were selected as targets for gene amplification analysis.

To analyze the gene amplification in an *in vitro* scenario, *L. infantum* promastigotes were made resistant to several antileishmanial compounds (MGA, MILT, TFL-A3 and TFL-A6) and the level of gene amplification accessed by real-time PCR. For absolute quantification by real-time PCR to be possible, the number of copies of each gene were normalized relatively to the number of copies of the single copy invariant gene for DNA polymerase alpha catalytic subunit (*POL1*, RefSeq XM\_001464606.1) for every sample analyzed.

By investigating the differences in gene copy number of the selected genes in the different resistant strains it is also possible to clarify the mechanisms responsible for the parasite survival when exposed to antileishmanial compounds.

### III. MATERIALS AND METHODS

#### 1. *In vivo* infection and treatment

##### 1.1. Animals and parasites

###### 1.1.1. Animals

A total of 65 female BALB/c *Mus musculus* mice were used in three independent experiments. All the animals were infected with *L. infantum*. For the first two experiments 20 mice were randomly divided into four groups of five mice each. One untreated control group and each of the other three groups treated with a different drug: STB, TFL-A3 and TFL-A6. In the third experiment 25 mice were randomly divided into five groups of 5 mice each. One untreated control group and each of the remaining four groups were treated with a different drug: STB, TFL-A3, TFL-A6 and ORZ. The animals were purchased and maintained in the IHMT animal facility according to the EU requirements (86/609/CEE) and Portuguese law (DR DL129/92 and Portaria 1005/92).

###### 1.1.2. Parasites

*L. infantum* zymodeme MON-1 (MHOM/PT/89/IMT151) was used to infect BALB/c mice in these experiments. *L. infantum* MON-1 was maintained in the laboratory by successive passages in BALB/c mice. The spleens of infected mice were extracted and homogenized with a tissue disaggregator with 50 µm separator screen (Medicons, Syntec International, Ireland) to isolate a suspension of single cell amastigotes. This cell suspension was added to Schneider medium (SCHN, Sigma-Aldrich, Germany) supplemented with 10% (v/v) of heat-inactivated (30 minutes at 56°C) fetal bovine serum (FBS, Sigma-Aldrich), penicillin-streptomycin (Biochrom, Germany) at 100 U/ml and 100 µg/ml respectively (complete SCHN medium), and incubated at 24°C. In axenic cultures the intracellular amastigotes change into free metacyclic promastigotes.

##### 1.2. Infection

Concentration (promastigotes/ml) from cultures of metacyclic promastigotes was calculated by optical microscopy using a Neubauer Chamber. The culture was centrifuged at 1800xg for 10 minutes and resuspended in the appropriate amount of

sterile saline solution (0.90% m/v of NaCl) in order to achieve a concentration of  $1 \times 10^7$  promastigotes/ml. Each mouse was intraperitoneally (IP) inoculated with 100  $\mu$ l of this solution.

#### 1.3. Treatment

Forty-five days post infection mice were randomly sorted into groups of 5 mice each. The control group was IP injected with 200  $\mu$ l of trehalose-citrate buffer (10 mM sodium citrate, 135 mM NaCl, 29 mM trehalose, pH 5.5) once a day for 10 days. Groups treated with TFL-A3 and TFL-A6 (dissolved in trehalose-citrate buffer with 5% Tween 80) were IP injected with a dose of 25 mg TFL-A/kg/day for 10 consecutive days. The group treated with ORZ (dissolved in trehalose-citrate buffer with 5% Tween 80) was intravenously injected for 10 consecutive days with a dose of 25 mg ORZ/kg/day. Fifty days post infection, the mice treated with STB were injected subcutaneously with 15 mg STB/kg/day for 5 consecutive days.

#### 1.4. Parasitic load determination

Three days after the conclusion of the treatment mice were sacrificed and their spleen aseptically removed and weighted. The spleens were homogenized individually in 4 ml of complete SCHN medium using Medicons. Viable parasite loads were estimated by limiting dilution assay (LDA) in 96 well plates. An additional 1:2 dilution of the cell suspension was made and 200  $\mu$ l of this cell suspension was placed in the first well of each row of 96 well plates and fourfold serial dilutions were made until the last column of the plate. For each homogenized cell suspension, four rows of a 96 well plate were used in order to have four replicates. The LDA plates were sealed and incubated during 15 days at 24°C. After incubation each well was examined by optical microscopy and labelled as positive if promastigotes were present or negative if no parasites were detected. The highest dilution for which the well contained promastigotes was used to calculate the number of parasites per gram of tissue (ppg) as follows:

$$\text{ppg} = \frac{\left( \frac{\text{Reciprocal titer of highest positive dilution} \times \text{Volume of cell suspension}}{\text{Volume of first well} \times \text{Dilution factor}} \right)}{\text{Weight of homogenized tissue (g)}}$$

The content of the highest positive dilution wells for each group was collected, washed three times by centrifugation at 1800xg for 10 minutes with PBS (Lonza, Belgium) and frozen at -80°C in PBS for posterior DNA extraction.

## **2. *In vitro* resistant parasites**

### **2.1. Parasites**

*L. infantum* zymodeme MON-1 (MHOM/PT/89/IMT151) was the strain used to generate drug-resistant promastigotes *in vitro*. *L. infantum* MON-1 was maintained in the laboratory as described in 1.1.2. and never had contact with any type of antileishmanial drug.

### **2.2. Drug-resistant promastigotes**

#### **2.2.1. Drug dilutions and plating**

Four antileishmanial drugs were used to generate *in vitro* drug-resistant promastigotes: MGA, MILT, TFL-A3 and TFL-A6. A solution of commercial Glucantime® (Merial, France) with 81 mg/ml of meglumine antimoniate and a solution of commercial Milteforan® (Virbac, France) with 20 mg/ml of miltefosine were used. These initial solutions were diluted to a concentration of 64 mM and 1 mM in SCHN medium, respectively. The purified TFL-A3 and TFL-A6 compounds were dissolved in a solution of DMSO/Ethanol (1:1) to a concentration of 65 mM. These initial solutions were then diluted in SCHN medium to a final concentration of 2 mM. For each of the compounds seven twofold serial dilutions were made in SCHN medium. A different dilution was used for each row of a 96 well plate. Each well was filled with 100 µl of the appropriate dilution. A control plate where all the wells were filled with 100 µl of SCHN medium was also performed.

Concentration (promastigotes/ml) of *L. infantum* culture was calculated by optical microscopy using a Neubauer Chamber as described in 1.2.. The culture was centrifuged at 1800xg for 10 minutes and resuspended in SCHN medium supplemented with 20% FBS in order to achieve a concentration of  $1 \times 10^8$  promastigotes/ml. Every well of the 96 well plates with the drug dilutions were filled with 100 µl of *L. infantum* culture. The plates were sealed and incubated at 24°C for 96 hours.

### 2.2.2. Evaluation of resistant promastigotes

After incubation, each well was examined by optical microscopy and labelled as positive if live promastigotes were present or negative if no live promastigotes were detected. The highest dilution for which the well contained live promastigotes was centrifuged at 130xg for 10 minutes to exclude all the dead promastigotes, the supernatant was collected and washed three times by centrifugation at 1800xg for 10 minutes in sterile saline solution and resuspended in 1 ml of complete SCHN medium. The concentration (promastigotes/ml) was calculated for each sample and the cultures were grown in complete SCHN medium with the drug concentration of the original well until enough promastigotes were present to repeat the steps in 2.2.1.. This continuous process took place until the highest dilution for which the well contained live promastigotes had double the drug concentration of the lowest dilution with no live promastigotes for the first time this process was done.

### 2.2.3. Sample collection

After the increase of drug resistance by fourfold, the content of the wells was collected and centrifuged at 130xg for 10 minutes to exclude all the dead promastigotes, the supernatant was collected and washed three times by centrifugation at 1800xg for 10 minutes in PBS and frozen at -80°C in PBS for posterior DNA extraction.

## 3. Primer selection and plasmid cloning

### 3.1. Primer selection and optimization

Using Primer-BLAST software [Ye *et al.*, 2012] appropriate primers were selected for the genes studied (Table 1). Primers (Stabvida, Portugal) were optimized by PCR changing the annealing temperature ( $T_{AN}$ ) and duration of cycles until the desired fragment was the only DNA amplified from a sample of *L. infantum* DNA, and no amplification whatsoever was observed for samples of mice (Figure 10). Initial  $T_{AN}$  of each gene fragment was calculated subtracting 5°C to the primer melting temperature specified by the manufacturer of the primers.  $T_{AN}$  was gradually increased by 0.5°C until the required amplification specifications were met. Amplification was done in 20 µl of a mixture containing 2 µl of sample, 2 µl of 10x NH<sub>4</sub> Reaction Buffer (Bioline, UK), 0.8 µl of 50 mM MgCl<sub>2</sub> solution (Bioline), 0.5 µl of 100 mM dNTPs solution (Bioline), 0.4 µl of

20 pmol/μl forward primer solution and reverse primer solution, 0.2 μl of 5 U/μl BIOTAQ DNA Polymerase solution (Bioline) and 13.7 μl of ultra-pure water. Optimized conditions obtained for amplification were 5 minutes at 95°C for complete DNA denaturation, 30

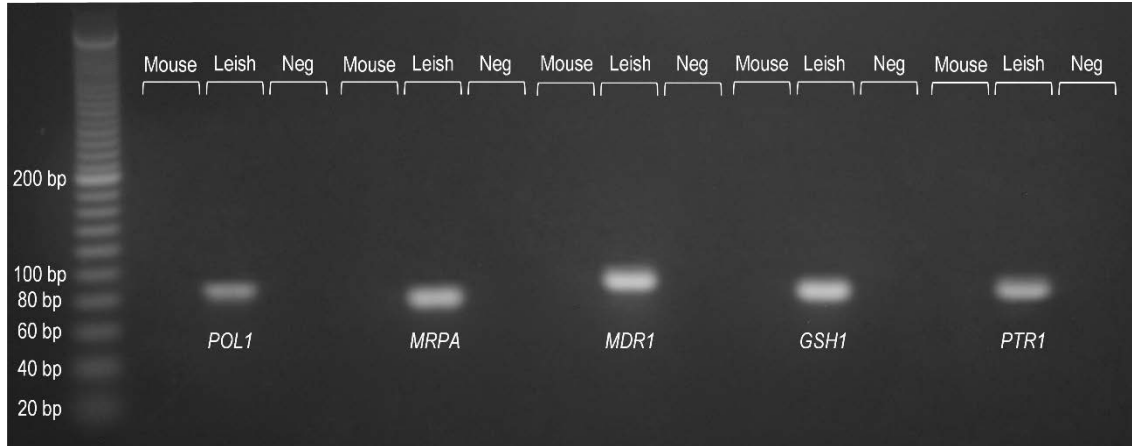


Figure 10. Electrophoresis gel showing the amplified fragments of the genes *POL1*, *MRPA*, *MDR1*, *GSH1* and *PTR1*. Wells were loaded with mouse DNA (Mouse), *Leishmania* DNA (Leish) and a control without DNA (Neg). *POL1* (*POL1*), *MRPA* (*MRPA*), *MDR1* (*MDR1*), *GSH1* (*GSH1*) and *PTR1* (*PTR1*) fragments were amplified at 86 bp, 76 bp, 89 bp, 84 bp and 86 bp, respectively.

cycles of 30 seconds at 95°C, 15 seconds at primer/gene specific  $T_{AN}$  (Table 1) and 10 seconds at 72°C, and a final step of 3 minutes at 72°C to guarantee complete elongation of most PCR products initiated during the last cycle. Samples were analyzed by DNA electrophoresis using a gel with 3% (m/v) agarose in Tris-acetate-EDTA (TAE) buffer (Merck, USA) containing 0.1 μl/ml of 10000x GelRed Nucleic Acid Stain (Biotium, USA).

Gene	Primers	Fragment Size	$T_{AN}$ (°C)
<i>POL1</i>	FW - 5' CTGCAGCGCCAAAGGTCTACCC 3' RV - 5' TCTTGCCGAGTTGCTGTGCGAG 3'	86 bp	62.0
<i>GSH1</i>	FW - 5' CCCGGCATTCTCTGGCTCTCAGC 3' RV - 5' GCGATAGTCAGCCAGCGCACAT 3'	84 bp	63.5
<i>MDR1</i>	FW - 5' GGAGGGTGACACAAGCGACACG 3' RV - 5' CGACGCGATCCGCCTTCATGTT 3'	89 bp	62.0
<i>MRPA</i>	FW - 5' CCGCTCGCGGACCACATTGT 3' RV - 5' TCGCCGCAAAGGCAGCGTAA 3'	76 bp	62.0
<i>PTR1</i>	FW - 5' ACGTGCTCGTGAACAACGCCTC 3' RV - 5' ATCTCCGACACAGGGCAGGTGG 3'	86 bp	65.5

Table 1. List of forward (FW) and reverse (RV) primers, base pair number (bp) of amplified fragment and primer annealing temperature ( $T_{AN}$ ) for each gene studied.

### 3.2. Plasmid cloning

Gene fragments were again amplified by PCR and 2 µl of the PCR product was analyzed by DNA electrophoresis to confirm amplification was done correctly. The remaining PCR product (18 µl) was purified using innuPREP DOUBLEpure Kit (Analytik Jena AG, Germany) following the manufacturer's instructions and 2 µl of the purified product was again analyzed by DNA electrophoresis. The remaining purified fragment product was introduced into a circular plasmid using pGEM®-T Easy Vector Systems (Promega, USA) as described in the manufacturer protocol. Competent *Escherichia coli* strain JM109 was used for plasmid cloning. *E. coli* JM109 was grown overnight in solid M9 medium (0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.1% NH<sub>4</sub>Cl, 0.2% MgSO<sub>4</sub>, 0.01% CaCl<sub>2</sub>, 0.4% Glucose and 1.5 % Agar in distilled water, all percentages in m/v) supplemented with 1 mM of thiamine. After incubation a colony was selected and grown in 5 ml of LB broth EZMIX (Sigma-Aldrich) overnight at 37°C. Five ml of overnight culture were inoculated into 500 ml of LB medium and left to grow at 37°C in a rotary shaker at 250 rpm until OD<sub>600</sub> reached 0.4. The bacterial culture was then pelleted by centrifugation at 1000xg for 10 minutes at 4°C and resuspended in 50 ml of ice-cold transformation and storage solution [TSS - LB with 10% (m/v) of PEG 8000, 5% (v/v) of DMSO and 50 mM of MgCl<sub>2</sub>] [Chung *et al.*, 1989]. Aliquots of this solution were prepared, frozen with liquid nitrogen and then placed on -80°C for posterior use. Frozen aliquots were then thawed on wet crushed ice and 50 µl of thawed competent cells transferred to 2 ml tubes previously chilled on ice. Two µl of plasmid with the gene fragment insert were added to these tubes and incubated on ice for 30 minutes. Tubes were then subjected to heat shock in a water bath at 42°C for 60 seconds and placed on ice again for 2 minutes. To each tube 500 µl of Super Optimal broth with Catabolite repression (SOC, Invitrogen, USA) was added and incubated at 37°C with shaking at 250 rpm for 3 hours, to allow the cells to express antibiotic resistance. 250 µl of the cell solution was then plated on LB agar (LB with 1.5% (m/v) of agar) supplemented with 100 µg/ml of ampicillin (Sigma-Aldrich), 80 µg/ml of X-Gal (Sigma-Aldrich) and 0.5 mM of IPTG (Sigma-Aldrich) and incubated at 37°C for 18 hours. The use of this specific *E. coli* strain and the pGEM®-T Easy vector allowed to differentiate the colonies containing the plasmid only from the colonies with the plasmid and the insert fragment by blue/white color screening and resistance to ampicillin. The white colonies were picked and incubated in LB supplemented with 100 µg/ml of ampicillin overnight at 37°C with shaking at 250 rpm. The isolation of plasmid DNA was then made using the innuPREP Plasmid Mini Kit (Analytik Jena AG) according to the manufacturer protocol. The plasmid DNA was



analyzed by PCR and DNA electrophoresis with the specific primers for the insert fragment in order to confirm its presence in the plasmid.

## 4. Quantification of gene number by real-time PCR

### 4.1. DNA extraction

DNA of the samples collected from *in vivo* and *in vitro* experiments was extracted using the innuPREP DNA Mini Kit (Analytik Jena AG) according to the manufacturer protocol and stored at -20°C for posterior use.

### 4.2. Real-time PCR

In order for absolute quantification to be possible calibration curves for each gene were made using the plasmids with the insert fragment described in 3.2.. The plasmid DNA was quantified and, for each gene 1:5 serial dilutions were made starting from 250 pg/μl to 0.016 pg/μl in ultra-pure water. A posterior 1:2 dilution of each of the previous dilutions was made in order to load the real-time PCR reaction mixture with 2 μl of each dilution instead of 1 μl. Samples collected as described in 1.4. and 2.2.3. were analyzed in triplicate for each gene by this technique. For each sample, 20 μl of the real-time PCR reaction mixture was prepared with 2 μl of sample, 10 μl of SensiFAST SYBR Lo-ROX (Bioline), 0.15 μl of 20 pmol/μl forward primer solution, 0.15 μl of 20 pmol/μl reverse primer solution and 7.7 μl of ultra-pure water. Amplification was done in an Applied Biosystems 7500 Fast Real-Time PCR System thermal cycler (Applied Biosystems, USA) and the conditions were 5 minutes at 95°C for complete DNA denaturation, 40 cycles of 30 seconds at 95°C and 30 seconds at primer/gene specific  $T_{AN}$  (Table 1) for annealing and extension, and 90 cycles of 10 seconds at starting temperature of 50°C with an increment of 0.5°C for each cycle. The fluorescence levels of each sample were analyzed in real time by the thermal cycler and the amount of fragment DNA calculated in comparison to the calibration curves for each gene. The number of copies was calculated as follows, were  $9.1 \times 10^{11}$  is the amount of DNA base pairs in 1 μg of DNA and 3015 is the length in bp of the plasmid used:

$$\text{Gene copy number} = \frac{9.1 \times 10^{11} \times \text{quantity } (\mu\text{g})}{3015 + \text{insert length (bp)}}$$

The results for each gene of each sample were divided by the copy number of *POL1* for the same sample, in order to normalize the results.

## 5. Statistical analysis

As stated before, three independent *in vivo* experiments were conducted for the drugs STB, TFL-A3 and TFL-A6. The statistical analysis was done using the non-parametric Mann-Whitney *U* test for two independent samples. The values obtained for parasitic load and gene copy number were compared between the study group (mice treated with STB, TFL-A3, TFL-A6 or ORZ) and the control group (not treated mice).

Three independent *in vitro* experiments were conducted for the drugs MGA, MILT, TFL-A3 and TFL-A6. The statistical analysis was done using the non-parametric Mann-Whitney *U* test for two independent samples. The values obtained for gene copy number were compared between the study group (promastigotes resistant to MGA, MILT, TFL-A3 or TFL-A6) and the control group (wild-type promastigotes).

All the data analysis was performed using the software IBM SPSS Statistics version 22.0 (IBM, USA). A significance level of 5% ( $p \leq 0.05$ ) was used to evaluate statistical significance of the data analyzed.

## IV. RESULTS

### 1. *In vivo* experiments

#### 1.1. Parasitic load

The control group, infected and not treated (Untreated) mice, for the experiments with the TFL analogues showed a mean value of  $1.18 \times 10^4$  viable promastigotes per gram of spleen. The group treated with STB presented a significant ( $p=0.022$ ) decrease of parasitic load ( $2.56 \times 10^2$  viable promastigotes per gram of spleen) pointing to 97.84% of parasite inhibition when compared to the Untreated group. Mice treated with TFL-A3 presented a reduction of 36.90% in parasite load, however, this reduction to  $7.48 \times 10^3$  viable promastigotes per gram of spleen was not statistically significant. Mice treated with TFL-A6 showed  $4.57 \times 10^3$  of viable promastigotes per gram of spleen pointing to a significant ( $p<0.001$ ) reduction of 96.14% in parasite burden (Figure 11).

In the experiment with ORZ the Untreated group presented  $1.48 \times 10^4$  viable promastigotes per gram of spleen. The treatment with STB and ORZ reduced parasitic load to  $2.56 \times 10^2$  ( $p=0.020$ ) and  $2.15 \times 10^2$  ( $p=0.014$ ) viable promastigotes per gram of spleen respectively, showing a 98.27% and 98.55% of inhibition in parasite replication (Figure 12).

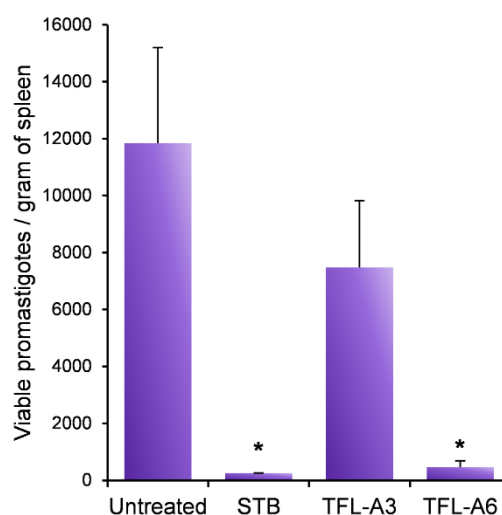


Figure 11. Parasitic load values on the mice treated with STB, TFL-A3 and TFL-A6. Viable parasites of untreated mice (Untreated) and of mice treated with STB (STB), TFL-A3 (TFL-A3) or TFL-A6 (TFL-A6) were estimated by limiting dilution assay. \* ( $p<0.05$ ) indicates statistically significant values when compared to Untreated group.

#### 1.2. Gene amplification

The parasites from untreated mice (WT), showed a mean of 1.29 copies of the gene *GSH1* (Figure 13a), 9.82 copies of *MDR1* (Figure 13b), 0.90 copies of *MRPA* (Figure 13c), and 0.76 copies of *PTR1* per parasite (Figure 13d). Parasites isolated from mice treated with STB presented a significant increase in the number of copies of *MDR1* ( $p=0.015$ , 21.14 copies per parasite) and *PTR1* ( $p=0.006$ , 1.09 copies per parasite),

when compared with WT. No significant differences were found for *MRPA* and *GSH1* genes. Parasites isolated from mice treated with TFL-A3 displayed a significant increase in the copy number of *MDR1* ( $p < 0.001$ , 24.74 copies per parasite) when compared with WT. No significant differences were noted for the genes *GSH1*, *MRPA* and *PTR1*. For the parasites of the group treated with TFL-A6 there was a significant increase in number for the genes *MDR1* ( $p < 0.001$ , 17.28 copies per parasite), *MRPA* ( $p = 0.005$ , 2.33 copies per parasite) and *PTR1* ( $p < 0.001$ , 1.31 copies per parasite), when compared to gene copy number of WT. No significant changes were seen for *GSH1*. For parasites that survived

the treatment with ORZ the results show an increase in copy number for the genes *GSH1* ( $p = 0.002$ , 1.97 copies per parasite), *MDR1* ( $p < 0.001$ , 15.87 copies per parasite) and *PTR1* ( $p = 0.006$ , 1.59 copies per parasite). No alteration was noted for the number of copies of *MRPA* (Figure 13).

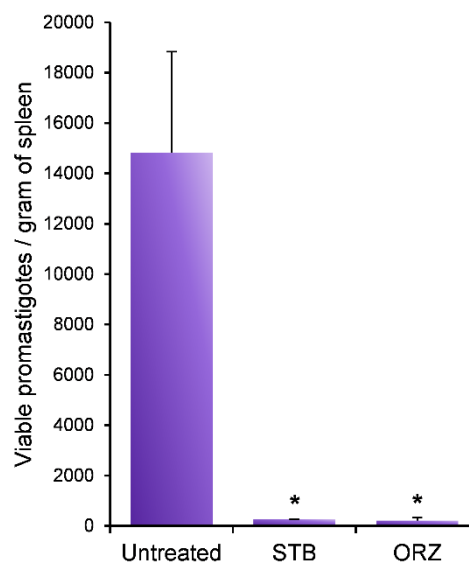


Figure 12. Parasitic load values of the mice treated with STB and ORZ. Viable parasites of untreated mice (Untreated) and of mice treated with STB (STB) or ORZ (ORZ) were estimated by limiting dilution assay. \* ( $p < 0.05$ ) indicates statistically significant values when compared to Untreated group.

## 2. In vitro experiments

### 2.1. Effective drug concentration

After the first 96 hours of incubation the effective concentration of the drugs able to kill all the parasites ( $EC_{100}$ ) was 8.00 mM for MGA, 15.63  $\mu$ M for MILT, 125.00  $\mu$ M for TFL-A3 and, 15.63  $\mu$ M for TFL-A6. After several cycles of exposure to the drugs the  $EC_{100}$  for each drug reached 32.00 mM for MGA, 62.50  $\mu$ M for MILT, 500  $\mu$ M for TFL-A3 and 62.50  $\mu$ M for TFL-A6.

### 2.2. Gene amplification

The wild-type parasites (WT) showed a mean of 9.97 copies of the gene *GSH1* (Figure 14a), 4.06 copies of *MDR1* (Figure 14b), 13.80 copies of *MRPA* (Figure 14c) and

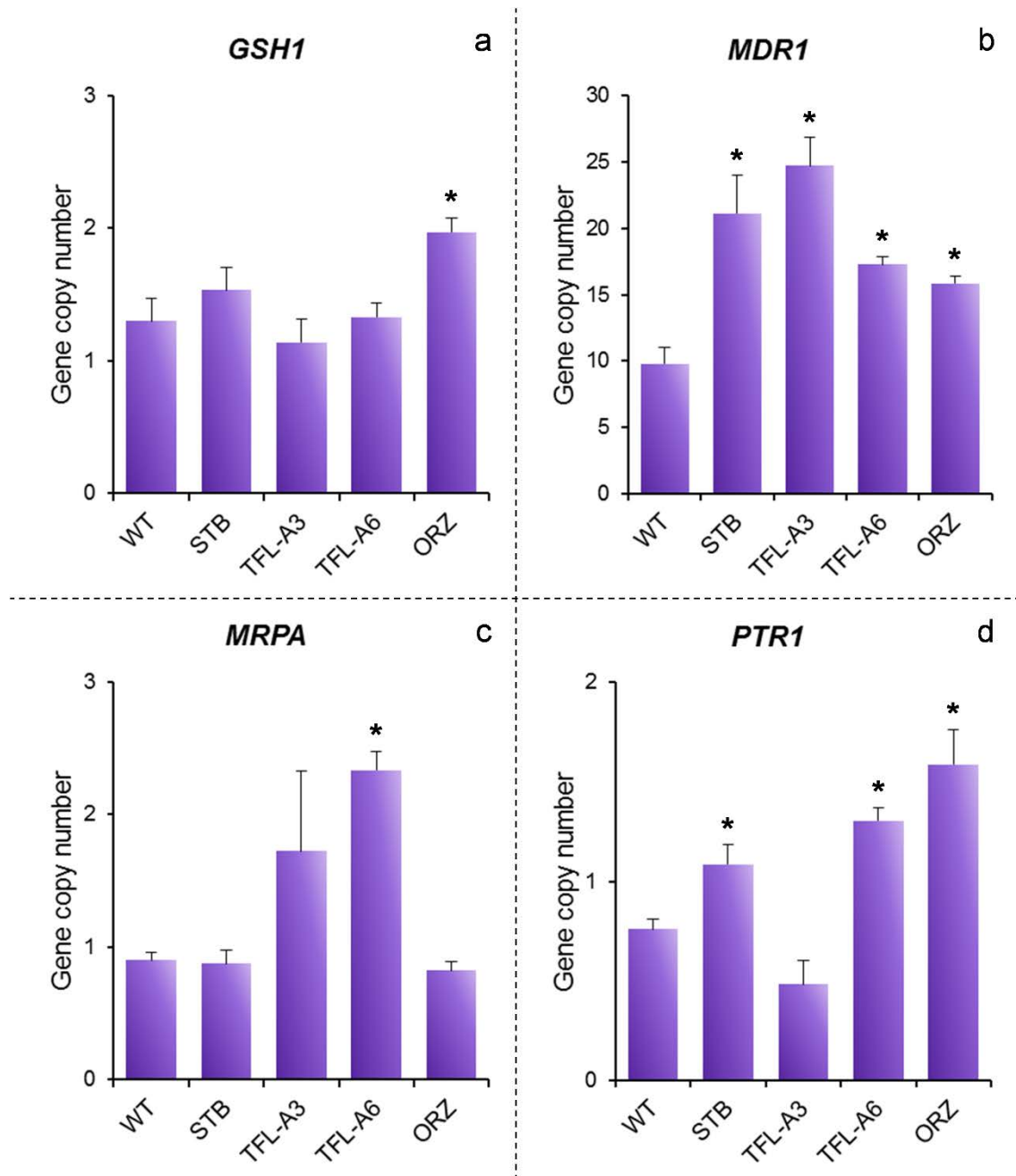


Figure 13. Mean of copy number of the genes *GSH1*, *MDR1*, *MRPA* and *PTR1* per parasite. Gene copy numbers of parasites isolated from mice treated with ORZ (ORZ), STB (STB), TFL-A3 (TFL-A3) and TFL-A6 (TFL-A6) and from non-treated mice (WT) were determined by quantitative real-time PCR. \* ( $p < 0.05$ ) indicates statistically significant values when compared to WT.

0.51 copies of *PTR1* per parasite (Figure 14d). The parasites made resistant to MGA displayed a significant decrease in gene copy number of the gene *MDR1* ( $p=0.001$ , 3.31 copies per parasite) and an increase in copy number of the gene *GSH1* ( $p=0.006$ , 11.99 copies per parasite) when compared with WT. No significant differences in copy number were found for the genes *MRPA* and *PTR1*. MILT-resistant parasites showed no significant alteration in copy number of the studied genes when compared to WT. On the other hand TFL-A3-resistant parasites presented a significant increase of *GSH1* copy number ( $p=0.003$ , 12.25 copies per parasite). No significant differences were noted for

the copy number of *MDR1*, *MRPA* or *PTR1*. TFL-A6-resistant parasites revealed a significant increase of *GSH1* ( $p=0.001$ , 17.72 copies per parasite), *MDR1* ( $p=0.003$ , 5.46 copies per parasite) and *PTR1* ( $p=0.001$ , 10.07 copies per parasite) when compared with WT. A significant decrease in *MRPA* copy number ( $p=0.004$ , 9.82 copies per parasite) was also observed (Figure 14).

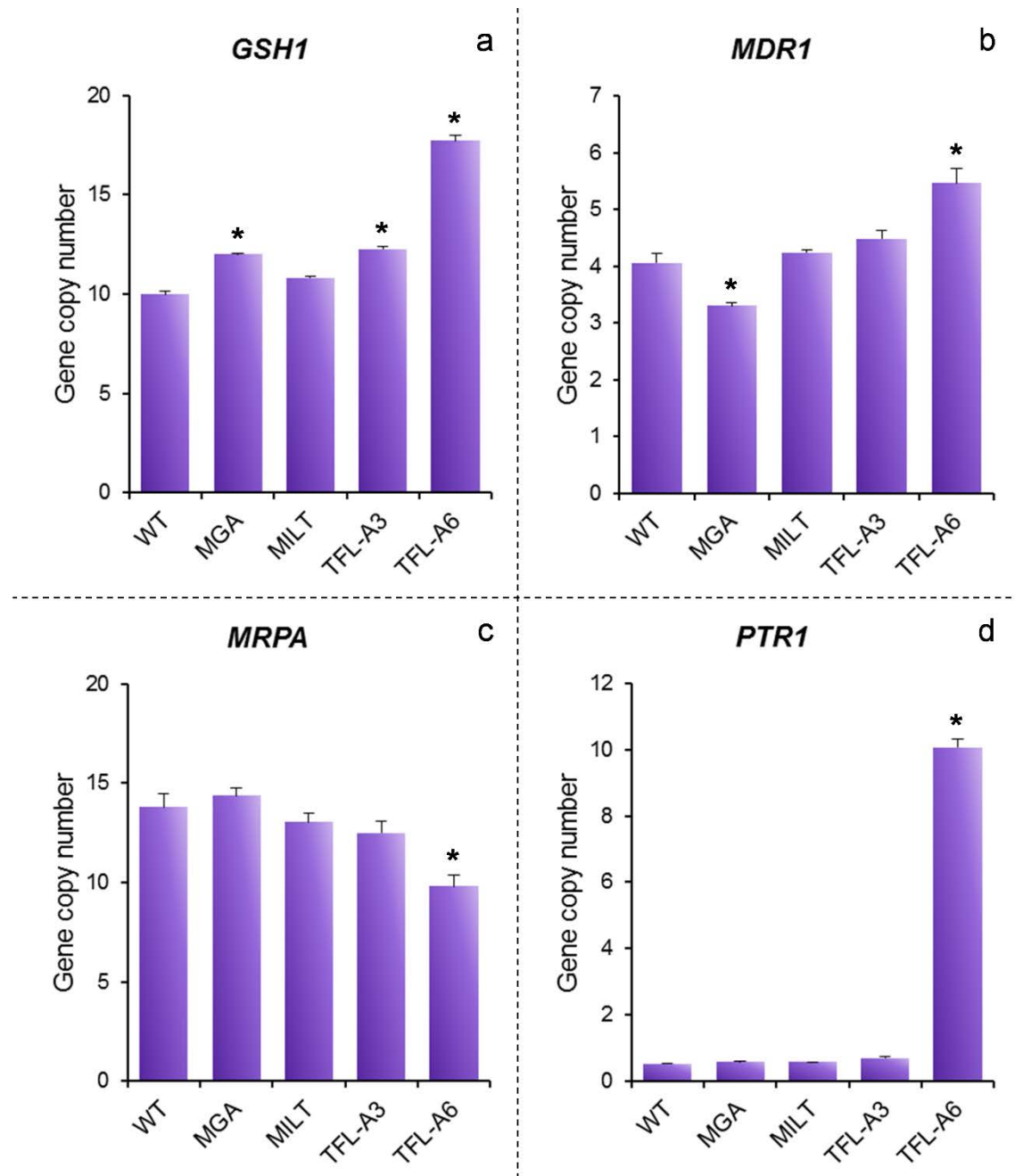


Figure 14. Mean of gene copy number of the genes *GSH1*, *MDR1*, *MRPA* and *PTR1* per parasite. Gene copy number of MGA (MGA), MILT (MILT), TFL-A3 (TFL-A3) and TFL-A6 (TFL-A6) resistant parasites (*in vitro*) and of wild-type parasites (WT) were determined by quantitative real-time PCR. \* ( $p < 0.05$ ) indicates statistically significant values when compared to WT.

## V. DISCUSSION

### 1. Antileishmanial activity of the drugs

From the drugs studied the one with the best antileishmanial activity and future potential is TFL-A6, although the results for ORZ seem also very promising.

TFL-A3 showed a weak antileishmanial activity *in vivo* and therefore its use in this formulation was ruled out. However, this low activity may be related to the delivery of the drug to the infected areas in the host and its internalization by the infected macrophages, since previous *in vitro* studies showed great antileishmanial activity against *Leishmania* [Esteves *et al.*, 2010].

The antileishmanial activity of TFL-A6 was comparable to STB (96.14% and 97.84% parasite reduction, respectively), a commonly used antileishmanial drug. TFL-A6 can be a promising drug to be used for the treatment of visceral leishmaniasis. However, as it happens for STB in animal models, this formulation was not able to kill all the parasites in the host which is a major concern because it may lead to the selection of resistant parasites. Similarly the treatment with ORZ was not able to clear all the parasites and again, the emergence of drug resistant strains from the surviving parasites should be strongly considered.

### 2. Gene amplification and mechanisms of drug action

By analyzing the DNA of *Leishmania* parasites that survived to treatment with STB, TFL-A3, TFL-A6 or ORZ it was possible to determine the number of gene copies of four genes associated with drug resistance, *MDR1*, *MRPA*, *GSH1* and *PTR1*, in the parasites. By surviving the treatment it was hypothesized that this parasite would have some sort of advantage in surviving the contact with the drugs used, being less susceptible or resistant to the drugs.

The parasites that survived the treatment with STB presented a significant increase in copy number of the genes *MDR1* and *PTR1*. *MDR1* is a gene responsible for the expression of the P-glycoprotein MDR1, which is required for the extrusion of drugs. The amplification of this gene is associated with resistance to several hydrophobic drugs, however, no direct relation to resistance to hydrophilic drugs, like Sb<sup>III</sup>, was demonstrated so far. The amplification of this gene was already reported for *L. infantum* isolated from clinical samples from VL cases resistant to antimonials. However, because of the clinical nature of the samples, there were doubts about if *MDR1* amplification was because of

other drugs used for treatment of other diseases in the patients [Mary *et al.*, 2010; Jeddi *et al.*, 2011]. In the present study, the group of mice treated with Sb<sup>III</sup> was not subjected to any other treatment, therefore we can conclude that MDR1 is involved in the efflux of Sb<sup>III</sup> and, by consequence, in resistance to this antileishmanial compound. The amplification of *PTR1* suggests that less sensitivity to STB can be associated to the folate metabolic pathways. *Leishmania* requires an exogenous source of folate and unconjugated pteridines to replicate and infect the vertebrate host. The enzyme PTR1 is responsible for the reduction of biopterin into H<sub>4</sub>-biopterin, which was also shown to play a key role in the biosynthesis of nitric oxide and to be connected to the sensitivity of *Leishmania* to oxidants [Nare *et al.*, 2009; Ong *et al.*, 2011; Vickers & Beverley, 2011]. These evidences lead to two possible conclusions. The amplification of *PTR1* may give an advantage in the surviving of the parasites inside the host macrophage by protecting against oxidative stress, and this way the amplification is not directly related to the drug used. On the other hand, *PTR1* amplification may decrease the efficacy of Sb<sup>III</sup> by reducing the reactive oxygen and nitrogen intermediates, making the inhibition of trypanothione reductase (TR) by Sb<sup>III</sup> less relevant (Figure 8).

TFL-A3-surviving parasites only present *MDR1* amplification. As stated before, MDR1 can be responsible for an increase in efflux of TFL-A3. This can be explained by the fact that the main targets of MDR1 are hydrophobic compounds and the low water-solubility of this drug suggests that it have a hydrophobic structure.

Three of the four studied genes (*MDR1*, *MRPA* and *PTR1*) showed significant amplification in the parasites that survived to TFL-A6 treatment. The resistance to this derivative seem to be associated with its accumulation inside the parasite and with oxidative/nitrosative stress. The ABC-thiol transporter MRPA was shown to be responsible for sequestering Sb<sup>III</sup>/thiols conjugates into intracellular organelles in the parasite. A lower concentration of Sb<sup>III</sup> was also found inside parasites with amplified *MRPA*. Although is not clear how this happens, it seems to be related to a decrease of drug influx and not active efflux, since mutants with amplified *MRPA* shown rates of Sb<sup>III</sup> efflux similar to wild-type parasites [Callahan *et al.*, 1994; Grondin *et al.*, 1997; Haimeur *et al.*, 2000]. Similarly, a decrease of TFL-A6 influx could also occur. This effect in conjugation with the survival advantages conferred by the increase of MDR1 and PTR1 indicate that the parasite can use different mechanisms to survive when under drug pressure.

Once again, the amplification of *GSH1*, *MDR1* and *PTR1* in the ORZ-treated parasites suggests an association of parasite resistance is related with the increase of drug efflux and high protection from oxidative/nitrosative stress. Furthermore, the amplification of *GSH1* denotes that the mechanism of action of this drug may also be related to the



alteration of redox balance inside the parasite, and by consequence the counteraction by the parasite involves the glutathione and trypanothione pathways (Figure 8).

In the *in vitro* experiments performed, the parasites that were made resistant to the drugs MGA, MILT, TFL-A3 or TFL-A6 shown different results when compared to the ones achieved in the *in vivo* experiments. This may be explained by the different developmental stages of the parasite in both experiments. While in the *in vivo* experiments the parasite contacted with the drugs when inside the macrophage in the amastigote form, in the *in vitro* experiments the parasite was in promastigote form. Differences have been reported in several cellular processes, like metabolism, intracellular transport and response to oxidative stress, between intracellular amastigotes and axenic promastigotes. Also the fact that some compounds, like  $\text{Sb}^{\text{V}}$ , need to be metabolized by the macrophage to get activity and that in some cases macrophages are the drug targets also contributes to these differences [Vermeersch *et al.*, 2009; De Muylder *et al.*, 2011]. The wild-type parasites alone shown a significant difference in gene copy number for the genes *GSH1*, *MDR1* and *MRPA* between the parasites from the *in vivo* and *in vitro* experiments (Figure 15).

For the made *in vitro* MGA-resistant parasites, significant differences were noted for the number of gene copies of the genes *GSH1* and *MDR1*. The increase of gene copy number for *GSH1* can be explained by the involvement of *GSH1* in the glutathione and trypanothione pathways, responsible for the redox balance of the parasite. This seems to be enough for the survival of the parasite when in contact with high concentrations of MGA. MGA, like all pentavalent antimonials, needs to be reduced to  $\text{Sb}^{\text{III}}$ . *In vivo* the reduction of  $\text{Sb}^{\text{V}}$  to  $\text{Sb}^{\text{III}}$  occurs mainly in the

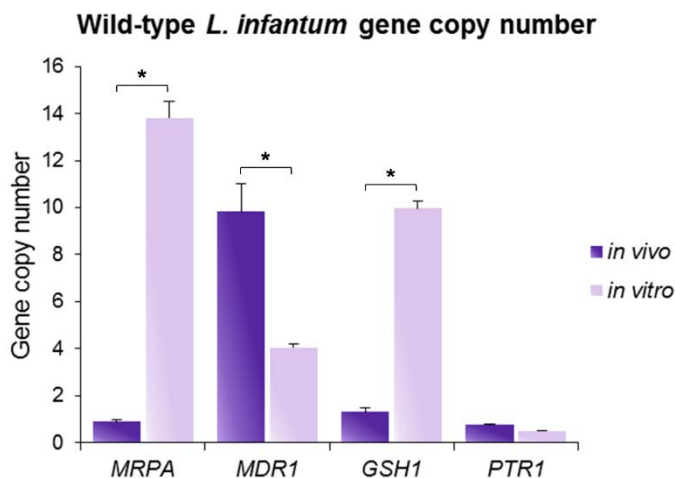


Figure 15. Comparison between the gene copy number of wild-type parasites for the *in vivo* and *in vitro* experiments. \* ( $p < 0.05$ ) indicates statistically significant values between the parasites of *in vivo* and *in vitro* experiments.

host macrophage. *In vitro* antileishmanial activity of this drug is low when presented to axenic promastigotes in culture. There is no direct explanation for the reduction in the number of gene copies for *MDR1*. This characteristic was most likely selected because it saves energy, otherwise wasted pumping out  $\text{Sb}^{\text{V}}$  from the parasite, which is not harmful unless reduced to the trivalent form.

For the parasites made resistant to MILT there was no differences in the gene copy numbers. This is most likely due to the fact that resistance to MILT appears mostly due to point mutations and not amplification. Also, amplification of genes other than the ones studied may be responsible for resistance to MILT.

The parasites made resistant to TFL-A3 only shown amplification for the gene *GSH1*. This, as stated before, is most likely related to the redox balance inside the parasite which seems to suffice for the parasite survival.

The *in vitro* TFL-A6-resistant parasites shown significant amplification for the genes *GSH1*, *MDR1* and *PTR1* associated with a significant decrease in copy number of *MRPA* gene. Amplification of *GSH1* and *MDR1* are connected to the maintenance of redox balance and efflux of the drug from the parasite, respectively. The high level of amplification of *PTR1* (ten times more than WT) in conjugation with the amplification of *GSH1* indicate that the mechanism of action of this drug in the promastigote is related to redox imbalance and oxidative stress. TFL-A6 resistance can also be connected with the availability of biopterins, by inhibiting biopterin transporters or its pathways, for example. It was shown before that H<sub>4</sub>-biopterin is essential for promastigote replication [Ong *et al.*, 2011]. The reduced number of *MRPA* gene copies seems to indicate that the accumulation of TFL-A6 inside intracellular organelles may be a disadvantage to the survival of the promastigote when in culture.

### 3. General conclusions and future perspectives

This study allowed to conclude that even though some drugs may demonstrate a good antileishmanial potential *in vivo*, their potential as good antileishmanial drugs may be less than ideal. The fact that a short-term period of treatment (10 days) was enough for such significant changes in the parasite DNA leads to the conclusion that in a wide-range drug-use scenario many cases of drug-resistant parasites may appear. Considering that amplification of the same genes is shared between the classical antileishmanial drugs (STB and MGA) and TFL-A6, the clinical use of this new compound may prove ineffective at short time.

These factors should all be taken into consideration when designing and testing new antileishmanial drugs. The techniques used in this study should be improved and the range of genes studied increased, achieving an effective screening method for the potential resistance mechanisms displayed by new drugs. This would not only save time when selecting new potential drugs but also prevent unexpected relapses of the disease and the emergence of less susceptible parasites due to drug resistance in the future.

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